A Dissertation for the Degree of Doctor of Philosophy

Immunomodulating Activity and Mechanism of 20-O-β-D-Glucopyranosyl-20(S)-Protopanaxadiol Fortified Ginseng Extract in Mouse Atopic Dermatitis Model

마우스의 아토피성 피부염 모델에서 글루코피라노실 프로토파낙사디올 강화 인삼추출물의 면역조절 효능 및 기작

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Major in Biomodulation

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By

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Abstract

Atopic dermatitis (AD) is a disease that distress to patient chronically accompanied by xerosis and pruritus. AD is not life-threatening disease; however, continuously pruritus and distress disturb sleep, and lead to impair the quality of life of the patients and their family. AD have been suggested that a major public health problem worldwide. Heredity, westernized life style, environmental pollution, modern diet, and drastic changes of climate have been suggested to cause developing of AD. The pathogenesis of AD is described by a two-phase, an initiation phase, which represents a Th2 polarized immune response and which develops without clinically apparent skin lesions, is switched into a second, eczematous phase, which is dominated by the Th1 cytokine interferon-γ and presents clinically as eczema. For several thousand years, human has been using various plants as food, medications, and cosmetics to improve the health and quality of life. Particularly ginseng (*Panax ginseng*, C.A. Meyer) has been a popular herbal remedy has been used for more than 4,500 years. Recent studies have demonstrated the adaptogenic effects of ginseng and ginsenosides for maintain homeostasis including immunomodulation. Based on previous results, I investigated that immunomodulation effects of 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD), which is known for most

effective ginsenoside, fortified ginseng extract (GFGE) in *Dermatophagoides farinae* extract induced atopic dermatitis (AD) model in NC/Nga mouse. According to these results, GFGE can prevent the development of AD, and improve the symptoms of AD, through inhibiting scratching incidence, inflammation, infiltration of eosinophils and mast cells. These results are confirmed by regulation of production of Th1/Th2 cytokine in splenocytes, and expression of transcription factors in skin lesions. These results indicate that GFGE can provide the preventative or sustainable management strategy for AD.

Key words: *20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD, compound K), ginseng, atopic dermatitis, Dermatophagoides farinae, NC/Nga*

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Chapter 1.

Regulation of atopic dermatitis by dietary phytochemicals: A review

Abstract

Atopic dermatitis (AD) is a disease that distress to patient chronically accompanied by xerosis and pruritus. AD is not life-threatening symptom; however, continuously pruritus and distress disturb sleep, and then lead to impair the quality of life of patients and their family. AD has been suggested that a major public health problem worldwide. Heredity, westernized life style, environmental pollution, modern diet, and drastic change of climate have been suggested to cause development of AD. The pathogenesis of AD is described by a two-phase, an initiation phase, which represents a Th2 polarized immune response and which develops without clinically apparent skin lesions, is switched into a second, eczematous phase, which is dominated by the Th1 cytokine interferon-γ and presents clinically as eczema. Plants including fruits, vegetables, and herbs are good sources of nutrient, which are used in beverages, cosmetics for maintaining the health or improving the quality of human life. Dietary phytochemicals include both allergy-promoting and anti-allergic nutrients. An appropriate intake of phytochemicals can prevent the development of allergic disease. Recent epidemiological and animal studies have demonstrated that ginseng, soy and other components in fruits and vegetables can ameliorate AD and other Th2 allergic diseases, such as allergic rhinitis and asthma. Collectively, these studies have been suggested that ginsenosides, soy isoflavone, and other

dietary phytochemicals in various fruits and vegetables can target the processes against allergic diseases, to regulate the immune imbalance. Therefore, regular consumption of proper phytochemicals could be a convenient way to maintain for immune homeostasis. Also, further studies will be needed for investigating the underlying molecular mechanisms involved, and then phytochemicals will be used as effective, safety alternative therapies.

1.1. Introduction

Atopic dermatitis (AD) is a disease that distress to patient chronically. It harasses and tickles patient chronically and repeatedly. AD is not a life-threatening symptom; however, continuous pruritus and distress can disturb sleep, and lead to impair the quality of life of patients and their families. AD is associated with other Th2 allergic disorders [1], which is regarded to bring out to develop allergic rhinitis or asthma through percutaneous sensitization [2]. In fact, AD is the most often first symptom of this atopic triad [2], therefore, early prevention of AD is very important.

AD is one of the most common skin disorders seen in infants and children [3]. Many reports have suggested that AD is a major public health problem worldwide, affecting around 5% to 20% of children at ages 6 to 7 and 13 to 14 years [4]. The past three decades have witnessed a marked rise in the prevalence of atopic dermatitis (AD) in some countries [5].

Clinical features of AD vary depending on the course of disease [6]. In acute phase, lesion is showed crusted, eroded vesicles or papules on erythematous plaques. And lesion with thick and excoriated in subacute phase, or with lichenified, pigmented, and excoriated plaques in chronic phase. Xerosis and pruritus are hallmark of AD [7].

AD had been divided into two forms; an “allergic” form associated with immunoglobulin E (IgE)-mediated sensitization involving 70–80% of

the patients, and an “nonallergic” form without IgE-mediated sensitization involving 20–30% of the patients [8, 9]. Recently, however, the World Allergy Organization (WAO) has revised that the terminology is classified into two categories; ‘atopy’ defined only associated with IgE-sensitization, and, ‘atopic disease’ is caused by IgE-mediated pathophysiology [7]. Thus the non-IgE-associated form (formerly intrinsic or atopiform dermatitis, or eczema) has to be distinguished from the IgE-associated form (formerly extrinsic). However, the non-IgE associated form have been referred to represent a transitional phase of the IgE-associated form, at least in infancy [7].

Although nonallergic form is not classified into atopic disease, T cells seem to be highly activated in both allergic and nonallergic form [10]. Cutaneous T cells that produce Th2 cytokines as IL-4, IL-5, and IL-13 predominate in the acute phase, whereas T cells that produce IFN- γ predominate in the chronic phase [11]. Although Th2 polarized immune response is explained the condition of AD generally, skin inflammation with initial Th2 phase is accompanied by chronic Tho/Th1 phase [12].

This review aims to summarize the broad pathogenesis involved in atopic dermatitis, and focuses on the important role of gene-environmental interaction, immunological imbalance and skin barrier disruption. I would like to discuss about immunomodulating activities of dietary phytochemicals

in widely-consumed fruits, vegetables, and herbs, inferring that simple dietary changes could have prevention or management of allergic diseases like atopic dermatitis.

1.2. Pathogenesis of atopic dermatitis

1.2.1. What causes atopic dermatitis?

Gene-gene, gene-environments interaction in atopic dermatitis

According to several studies, the incidence of AD is higher among monozygotic twins (77%) than among dizygotic twins (15%) [13]. Several genes have been identified in AD, notably chromosome 1q21 (associated to epithelium-related gene) [14], 5q31-33 (related to encode cytokines, interleukin (IL)-4, IL-5, IL-12, IL-13, and granulocyte-macrophage colony stimulating factor) [15, 16]. The filaggrin gene (*FLG*) on chromosome 1q21.3 encodes a key protein in epidermal differentiation [17], several loss-of-function mutations or distinctive *FLG* mutation were reported in European and Japanese patients [18-22]. The defect in the *SPINK5* gene (mapping to chromosome 5q31) is operative in patient with Netherton syndrome [23], and it is also showed in patient with AD [24]. Although family history has been distinctly concerned in AD susceptibility, genetic

factor alone cannot explain the increase in AD prevalence [25, 26].

Current studies have demonstrated that immigrants have increased risk of AD than those who have recently immigrated, with more prevalent in western industrialized countries than in developing countries [4, 27-29]. And the risk of AD is higher in children grown in smaller families, especially with higher socioeconomic status in urban [30-32]. Highest prevalence for AD (above 15%) were estimated in urban Africa, the Baltics, Australasia, and Northern and Western Europe, whereas lowest prevalence (under 5%) were present in China, Eastern Europe, and Central Asia [4]. These results suggest that an environmental etiology related with a ‘western lifestyle’ is important for development of AD. Therefore, AD arises by complex gene-gene and gene-environment interactions [12].

The hygiene hypothesis, hapten-atopy hypothesis, dietary hypothesis, and others

The hygiene hypothesis has been explained as a reason of rising onset of AD [33]. The main concept is the improvements in public health and hygiene affect the type and level of stimulation from microbial environment. So it might have influenced the development of immune function, then predispose to AD, however, this concept is still debated.

Modern diet might accelerate the development of AD [34, 35]. The

modern diet, specially westernized countries, is dominated by food, which has been processed, modified, stored for long time, whereas traditional diet is composed of unprocessed, shortly-eaten food [35]. And the nutrient content including minerals have changed by developing agricultural techniques for increasing storage period. These dietary changes have been considered to provoke to development of allergic disease associated with AD [36-38].

The hapten-atopy hypothesis suggests that hapten exposure is closely associated to AD [39] . In modern diet, there is artificially increased hapten chemicals in processed food, antibiotics, and other additives whereas only low amount of naturally occurring hapten in traditional diet. Therefore dietary hapten tolerance is dominant, with defective tolerance to natural food protein [40]. Actually, the increased exposure of haptenic properties of antibiotics, both in humans and farming livestock, also has coincided with the rise of AD [41].

The reduced natural antioxidant (from reduced intake of fresh, unprocessed vegetables) consumption might lead to increased permeability of the mucosa to allergen, and increased inflammation with altered Th1/Th2 balance [36].

The lipid hypothesis suggests that decreased consumption of fish and butter with increased uptake of certain polyunsaturated fats has led to lipid imbalance of membranes of inflammatory cells, which suppress Th1

differentiation [38].

Vitamin D hypothesis is that vitamin D might inhibit production of Th2 cytokines [42], as a result therapeutic use of vitamins D has been considered for AD patients [43-46]. However, on the contrary, recent several studies have demonstrated that administration of vitmain D might exacerbate AD and other Th2 allergic disease by promoting expression of thymic stromal lymphopoietin (TSLP) in skin and lung [47-50].

And many studies suggest that climate may affect the prevalence of AD, and Th2 allergic disease [51]. Abrupt climate and temperature shift may exert a bad influence to AD, itching significantly depends on meteorogical conditions and a certain range of thermohygric environments is essential for maintaining skin homeostasis [52, 53].

1.2.2. The pathogenesis of AD

AD is a chronic inflammatory skin disease with a pathogenesis of complicated immune imbalance and interaction of genetic, environmental and psychological factors [54]. A complex interplay of genetic, environmental, skin barrier function, psychological and immunological factors are involved in pathogenesis of this disease [55].

Cytokines and chemokines orchestrate atopic dermatitis; Th1/Th2 paradigm and its role in AD

The Th cell differentiation is dictated by the type of dendritic cell and microenvironment; on antigen presentation, IL-12 or IL-4 polarized naïve T cells to Th1 cells or Th2 cells [12].

The pathogenesis of AD could be best described by a two-phase model, in the acute phase of AD, Langerhans' cells are activated by allergens, and allergen-derived peptides are polarized T cells to Th2 profile [12]. And then recruited monocytes differentiate into inflammatory dendritic epidermal cells (IDEC) and produce the proinflammatory cytokines (IL-1 and TNF-α). Their secretion contributes to the switch from Th2 to Th1/0 response and leads to chronic phase of AD [12]. Although Th2 polarized immune response is an important the pathogenesis of acute phase in AD [56, 57], many observations suggested that the majority of house dust mite allergen specific T cell clones derived from the peripheral blood of AD patients produce both Th1 and Th2 cytokines [58-61]. The scratching induces the mechanical injury, resulting in proinflammatory cytokine and chemokine (CCL27) secretion [62]. Subsequently, chemokines recruit leukocytes to the skin, the leukocyte activation results in the release of inflammatory mediators, including effector cytokines (IL-31) and protease and neuropeptides which induce pruritic signals [62]. The sequential activation of Th2 cells following

Th1 cells, and various chemokines contribute the development of AD skin lesions, which take into condiseration of the different stages of disease [62].

Skin barrier defect in atopic dermatitis

The epidermis plays the critical defensive functions, such as permeability-barrier [63, 64] and antimicrobial-barrier [65]. The permeability-barrier abnormality in AD has been reported to important for disease activity [66]; because the permeability-barrier abnormality parallels severity of AD, emollient is useful for effective supportive agent, and the ceramide-dominant emollient therapy for the targets the lipid abnormalities improved the permeability-barrier dysfunction and inflammation of AD [67]. The sustained barrier defects (by decreased ceramide, loss-of-function of *FLG*) can induce Th1/Th2 dysregulation by the excessive exposure of allergen, and following decreased antimicrobial peptide cause the colonization of *Staphylococcus aureus*, which can accelerate inflammation and pruritus, and resulting the development of AD [66].

1.3. Current and potential therapeutics for treatment of atopic dermatitis

Ultimately, multifarious approach, including avoidance of various

allergens, which can induce inflammation and pruritus is demanded for successful management of AD [68]. The skin hydration and use of emollients, especially ‘ceramide-dominant’ emollient, for repairing of impaired skin barrier function is important an ancillary therapy [67]. If *Staphylococcus aureus* colonization can trigger AD, use of antibiotics can lead to a better condition [69].

Topical and systemic by cases, corticosteroids are the mainstay of anti-inflammatory treatment, showing effective function in acute and chronic skin inflammation [70] . These mediate anti-inflammatory effects through a cytoplasmic glucocorticoid receptor (GCR) in target cells, and ligand bound GCR binds to various transcription factors, including activator protein-1, NF-kB, via protein-protein interactions to inhibit the transcriptional activity of proinflammatory genes encoding proinflammatory proteins, such as cytokines(IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, TNF-a, GM-CSF), chemokines (IL-8, RANTES, monocyte chemotactic protein (MCP)-1, and eotaxin), and adhesion molecules (ICAM-1, V-CAM-1, and E-selectin) [71]. However corticosteroids also carry a risk of side effects; such as, hyperglycemia, insulin resistance, hypertension, osteoporosis, cataract, retinopathy, and colitis.

Topical calcineurin inhibitors, tacrolimus and pimeclorimus, are FDA-approved drugs for treatment of AD. These drugs inhibit the

calcineurin activity, and then nuclear factor of activated T cell protein (NF-ATp) is not dephosphorylated and cannot activate transcription of various Th1 and Th2 cytokine genes [72]. Many clinical studies have shown tacrolimus and pimeclorimus to be effective and quite safe drugs [72-74], even though these drugs had been reported that treatment for prolonged periods can lead to increased risk of cancer [75, 76].

Cyclosporin A (CsA) is a potent systemic calcineurin inhibitor. Many studies suggest that it is an effective drug in severe AD [77, 78]. However CsA should be used with caution because it can cause serious adverse effects, such as hypertension and renal toxicity [77].

There are several beneficial effects on AD by UV radiation treatment, which target to Langerhans cells or keratinocytes, interfering production of cytokines. Many studies demonstrate that high-dose UVA-1 is useful on acute, severe phase, and 311 nm UVB or low-dose UVA-1 is effective on chronic, moderate AD patients [79-81].

Besides that, other therapeutic approaches can be considered to restore Th1/Th2 immunobalance, by enhancing of Th1 response, or inhibiting of Th2 response (anti-IL-4, and anti-IL-13) [70]. In fact, the clinical studies using recombinant IFN- γ have been reported to improve patients' AD symptom [82-84]. In addition, anti-IgE antibodies, allergen-selective immunotherapy, and, chemokine antagonist for blocking of

inflammatory cell recruitment are applied for treatment to AD [71].

1.4. Immunomodulation and improvement effects of dietary phytochemicals in atopic dermatitis and related allergic diseases

Foods have been known to include both allergy-promoting and anti-allergic nutrients. Food allergy is occurred by specific components or ingredients (typically proteins, but sometimes chemical haptens). Food oils, such as soy, corn, peanut, and sesame, have broad ranges from very low allergenicity (in case of virtually all of the food protein removed in processing) to very high allergenicity (in case of little of the food protein removed in processing) [85]. Zuidmeer *et al*. reported that the prevalence of plant food allergies through systematic meta-analysis, including with 250,000 children and adults. However, only 6 studies were included for this report to analysis of plant food allergy, because the diagnostic gold standard were used only those studies since 1990. Prevalence estimates were categorized by food item (fruits, vegetables/legumes, tree nuts, wheat, soy, cereals, and seeds) and diagnostic method used (food challenges, skin prick test, serum IgE, parent/self-reported symptoms) [86]. Certain fruits and vegetables (strawberries, citrus fruit, and tomatoes) are considered to

stimulate the tissue mast cells to release histamine, then cause to allergic reaction, whereas specific IgE to these foods are not elevated [87] . The authors suggested these suspected allergic reaction to plant foods should be confirmed with double blinded, placebo-controlled challenge tests [86]. In this article, I will propose the possibility that an appropriate intake of phytochemicals with anti-allergic activity may provide an effective prevention strategy and an alternative therapy.

1.4.1. *Actinidia arguta* extract

Actinidia arguta, also referred to hardy kiwi, is native to Korea, China, Japan and Siberia, which has used as traditional remedy in China to improve general health for long time [88]. Park et al. has revealed that *Actinidia arguta* extract (from the hardy kiwi fruits) (*A. arguta*) has various anti-allergic effects. *A. arguta* extract has shown the inhibitory activity on production of IgE in U266B1 cells, and Th2 cytokine, IL-4, in ovalbumin stimulated splenocytes removed from mice. *A. arguta* has been also reported to increase the lowered level of Th1 cytokines (IL-12, IFN-γ), reduce the increased level of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) in ovalbumin-sensitized mice model. *A arguta* extract have been reported to contain immunobalancing effects were confirmed by reducing the IgE and IgG on plasma level [89]. These immunomodulation effects are confirmed on

spontaneous and DNCB-induced atopic dermatitis mouse models and on ovalbumin-induced asthma mouse model [90-92]. And also significantly reduced the levels of chemokines, such as eotaxin, eosinophil counts, and IgE, which is important mediator in human peripheral blood of allergic disease [93]. Based on these immunomodulation effects, *A. arguta* extract has been certified by Korean Ministry of Food and Drug Safety, and developed as commercial functional food.

1.4.2. Persimmon leaf extract

Persimmon (*Diospyros kaki)* is a kind of plant native to China, Korea, and Japan, and persimmon leaf is commonly consumed as beverages in Asia [94]. Persimmon leaf have been reported to have beneficial effects including hemostasis [95], hypertension [96], and hyperlipidemia [97]. Flavonoid including catechin, kaempferols, and quercetin, tannins, phenols, vitamin C *et al*. are found in persimmon leaf [94]. Persimmon leaf, and astragalin have been shown to preventive effects in AD mice model, through inhibit the histamine release, the scratching behavior and serum IgE elevation and transepidermal water loss (TEWL)[95, 98]. According to epidemiological studies, intake of persimmon leaf extract, included astragalin, quercetin 3-glycoside, kaempherol 3-galactoside, could decrease the scoring atopic dermatitis (SCORAD) index, eosinophil in peripheral

blood in patient of AD [99].

1.4.3. Evening primrose oil

Evening primrose (*Oenothera* spp.) is a plant native to America, and it also grows in Europe [100]. Evening primrose oil (EPO) has been reported to have beneficial activities such as improving rheumatoid arthritis [101-103], nerve function [104, 105]. Furthermore, several double-blind controlled cross-over studies demonstrated that in the EPO administered group, a statistically significant improvement was observed in the overall severity involved by dryness and itch, compared to placebo group [106-108]. Gamma-linolenic acid, the delta-6-desaturase metabolite of linolenic acid, have been known as effective component in evening primrose oil [109]. The efficacy of gamma-linolenic acid has been investigate in a lot of studies, generally gamma-linolenic acid was found to be effective to adults, children, and infants without side-effects double-blind, placebo-controlled studies [110, 111].

1.4.4. Soybean extract and soy isoflavone

The amount of soy products consumed in Asian countries, especially Korea and Japan is much greater than Western countries. Many studies demonstrates that soybean component and soy isoflavone have the beneficial effects on cancer [112], metabolic diseases, such as obesity, diabetes mellitus,

cardiovascular disease [113-115] and bone density [116].

Isoflavones are the most abundant components in soybeans, and present in various other beans, sprouts, and clover [112, 117]. Isoflavones are mostly contained as glucosides (daidzin, genistin) in nature, and on ingestion, the aglycones (daidzien, genistein) are produced by intestinal microorganism [117, 118]. Several studies have reported that soybean extract or isoflavones have anti-allergic effect [119-121]. A survey has been carried out in 1002 pregnant women to determine the impact of mar aglycones, daidzein and genistein on allergic rhinitis. These result revealed that consumption of soy products (tofu, cooked soybean), containing an abundance of isoflavones, significantly reduced symptom of allergic rhinitis [122]. Genistein has been found to have the anti-AD effect in NC/Nga mice [123]. Furthermore, according to our unpublished data, 7,3',4'-trihydroxyisoflavone could suppress the symptom of AD, and reduce the TEWL.

1.4.5. Ginseng extract and ginsenosides

For several thousand years, human has been using various plants as food, medications, and cosmetics to improve the health and quality of life. Particularly, Ginseng (*Panax ginseng*, C.A. Meyer, Araliaceae) has been a popular herbal remedy has been used in Asian for more than 4,500 years

historically, and it is first recorded about 2,000 years ago [124]. The distribution and consumption of ginseng have been taken globally [124, 125]. Moreover various beneficial effects of ginseng are revealed by clinically, and scientific researches in recent decades. The major active components of ginseng have been reported which is triterpene saponins, referred as ginsenosides [126] .

Many studies demonstrated the adaptogenic effects of ginseng and its ginsenosides to maintain homeostasis. Ginseng and ginsenosides have been reported to enhance the endocrinological activity under stress [127, 128], regulate level of blood glucose [129], improve the insulin resistance [130, 131]. In addition, they have been suggested to exhibit the anti-tumorigenic activity [132], chemopreventive effect [133, 134], cardiovascular protection [135, 136], and immune modulation activity [137-140].

In this review, I have focused on anti-allergic effect of ginseng and ginsenosides. Choo et al. reported that ginseng, ginsenoside Rb1, Rb2, Rc, Rd, F2, and 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD) have the inhibitory effect on β-hexosaminidase release from RBL-2H3 cells, and histamine release from rat peritoneal mast cells. And also, Ginseng, Rb1, F2, and GPD have shown to inhibiting effect on passive cutaneous anaphylaxis reaction. These inhibitory effects have shown to be more effective than

antiallergic drugs, disodium cromoglycate and azelastine [141]. The red ginseng, which manufactured by steaming and drying of raw ginseng at high temperature repeatedly, have been reported to ameliorate the atopic like symptom in mice through immunobalancing of Th1/Th2 response, regulating transcription factors, and inhibiting scratching behavior [142-146]. Rb1 and its metabolite, GPD, have been revealed to have the antipruritic effect in mice [147, 148]. Ginseng and ginsenosides also have the immunomodulation effect in other Th2 allergic disease, allergic rhinitis, and asthma in human, and mouse [149, 150].

1.4.6. Other dietary phytochemicals

The vegetarian diet, consists of spinach, cabbage, and sesame, which has been reported to ameliorate the symptom of AD patient through reduction of eosinophil and PGE2 synthesis [151]. According to this vegetarian diet, various flavonoids, 17 mg of apigenin, 1.6 mg of luteolin, 19.5 mg of quercetin, and 29 mg of kaemferol were consumed daily. Apigenin, luteolin, and fisetin have been reported to inhibit Th2 cytokine production in activated human basophils [152], apigenin attenuated exacerbation of AD symptom [153]. And also in mouse asthma model, apigenin and luteolin have been reported to decrease the inflammatory cell infiltration, airway hyperreactivity, and bronchoconstriction [154-156].

Topical administration of tannic acid and quercetin have suppressed the serum level of thymus and activation-regulated chemokine (TARC) and IgE in mouse [157].

According to epidemiologic studies demonstrates that high intake of polyphenol-riched foods (fresh fruit, vegetables, red-wine, and coffee) were negatively associated with prevalence or severity of allergic disease may protect against allergic disease [158]. And cocoa was suggested to inhibit IgE production in rat allergy models [159, 160]. Apple polyphenol was reported to be helpful to inhibit the develop of allergic diseases [161, 162].

Sulforaphane, and sulforaphene (our unpublished data) with in isothiocyanate group, have been known to suppress chemokine *in vitro* [163], and sulforaphane have been reported to inhibit the Th2 response in asthma model [164].

Until now, various dietary phytochemicals can be helpful to regulate allergic diseases including AD. With this, an appropriate intake of phytochemicals from fruits and vegetables is proposed to prevent and attenuate AD and other allergic diseases.

1.5. Conclusion

Although there are several drugs available for the treatment of AD, they should be used with caution because it can cause mild to serious adverse

effects, especially when chronic treatment is needed. AD is one of the most common skin disorders seen in infants and children, affecting around 5% to 20% of children. And several studies have demonstrated that approximately half of AD patients will develop asthma and two third will develop allergic rhinitis. Epidemiological studies have revealed that maternal AD poses a higher risk for infantile AD whether this may be due to genetic or congenital factors or both are uncertain. Therefore, prevention and continuous management of AD must remain a priority. The effective regulation of intermediates involved in production of Th1/2 cytokines, chemokines, and inflammatory damage represents a logical protection strategy. Various dietary phytochemicals in ginseng, soy, fruits and vegetables are known to be effect in modulating immune response, thereby ameliorating allergic diseases. Therefore, it stands to reason that regular consumption of dietary phytochemicals could be beneficial for the maintenance of immune homeostasis and protection against allergic diseases.

1.6. References

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Chapter 2.

20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol fortified ginseng extract prevents the development of atopic dermatitis

Abstract

Atopic dermatitis (AD) is chronic, pruritic inflammatory skin disease with a complicated pathogenesis of immune dysregulation, genetic, and environmental factors. Especially, AD is one of the most common skin disorders seen in infants and children, and it can trigger to develop other Th2 polarized diseases. So, safer and more effective therapy is required. Ginseng and ginsenosides are popularly employed in the treatment of chronic inflammatory diseases. Recently, 20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol (GPD), a main metabolite of ginsenosides, has been reported to have superior anti-allergic and anti-pruritic effect. This study was performed to assess the prevention effects of 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD) fortified ginseng extract (GFGE) on the development of AD in NC/Nga mice. Oral administration of GFGE significantly attenuated AD-like symptoms measured by dermatitis score, ear thickness, and scratching time in NC/Nga mice. GFGE treatment reduced the infiltration of eosinophils and mast cells in skin lesions. The beneficial clinical feature of GFGE was confirmed by the reduced production of Th1 and Th2 cytokines in splenocytes. These data suggest that GFGE might prevent the development of AD.

Key words: *Atopic dermatitis; Ginseng, 20-O-β-D-glucopyranosyl-20(S)-*

protopanaxadiol (GPD); Dermatophagoides farinae; NC/Nga; Th 1/2 cytokine; Immunobalance

2.1. Introduction

Atopic dermatitis (AD) is chronic, relapsing and pruritic inflammatory disease of skin [1]. Skin lesions associated with AD involve severe rash, edema, hemorrhage, and desquamation [2]. Pathological changes associated with AD include epidermal thickening and marked infiltration of inflammatory cells, such as eosinophils and mast cells [3]. Several observations suggest that AD is the cutaneous manifestation of a systemic disorder that also gives raise to allergic rhinitis, food allergy, and asthma [1, 4, 5]. Although AD is not life-threatening, it clearly requires early treatment and the management to prevent recurrence [6].

A predominant T-helper (Th)2 immune response with increased immunoglobulin (Ig)E production is widely recognized as distinct phenomena in the pathogenesis of AD, and Th2-mediated cytokines including interleukin (IL)-4, IL-5, and IL-13, are found in the skin lesion during the acute phase of AD [7]. On the other hand, during the chronic phase of AD, the dysregulated immune responses were accompanied by the combination of Th2 and Th1 responses [7]. Increased level of interferon (IFN)-γ, IL-12, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are observed in the chronic skin lesion of AD [7]. AD also accompanies mechanical tissue injury, resulting in the production of pro-

inflammatory cytokines including tumor necrosis factor (TNF)-α and GM-CSF [8].

Ginseng (*Panax ginseng* C.A. Meyer) has been frequently used as a traditional remedy [9]. The major components of interest in ginseng are ginsenosides, which contain an aglycone with a dammarane skeleton [9, 10]. The pharmacological actions of these ginsenosides have been explained on the basis of the biotransformation of ginsenosides by human intestinal bacteria [9, 11, 12]. Ginseng and its ginsenosides have been found to have anti-inflammatory, immunomodulatory and anti-allergic activity [9, 13, 14].

It has been reported that 20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol (GPD, also called as compound K), a main metabolite of ginsenosides Rb1, Rb2, and Rc transformed by human intestinal microflora, have anti-allergic activity [9, 15, 16]. GPD most potently inhibited β-hexosaminidase release from RBL-2H3 (rat basophil leukemia) cells and passive cutaneous anaphylaxis reaction in mice [9]. The inhibitory activity of GPD was more potent than that of sodium cromoglyate, one of the commercial anti-allergic drugs [9]. GPD also showed to have the anti-pruritic effect in scratching behavior of mice induced by compound 4/80, substance P, and histamine [17]. These observations suggest that GPD might be beneficial to prevent AD.

Therefore, I tested the effect of GPD fortified ginseng extract

(GFGE), which contains 8.2% of GPD, in *Dermatophagoides farinae* body extract (DFE)-induced AD-like symptoms in NC/Nga mice. Dermatitis score, ear thickness, scratching time, and histological change in skin were measured. The effects of GFGE on the production of Th1 and Th2 cytokines in splenocytes were also examined.

2.2. Materials and Methods

2.2.1. Chemicals and reagents

20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol fortified ginseng extracts (GFGE) is produced by CJ Food R&D (CJ CheilJedang, Seoul). AD Biostir cream, *Dermatophagoides farinae* extract (DFE), is obtained from Biostir (Hiroshima, Japan). Tacrolimus (0.1% Protopic® Ointment) is from Astellas Pharma (Tokyo, Japan). Isoflurane is from Hana Phram (Seoul, Korea). Hair removing cream is purchased from Reckitt Benckiser (Cedex, France). Sodium dodecyl sulfate is from Sigma (St. Louis, MO, USA). RBC lysis buffer is from Qiagen (Hilden, Germany). RPMI 1640 media is from Welgene (Daegu, Korea). FBS is from Sigma. Anti-CD3 mAb, and anti-CD28 mAb are from BD Bioscience (San Jose, CA, USA). RIPA lysis buffer is from Cell Signaling Biotechnology (Danvers, MA, USA). EDTA-free protease inhibitor cocktail is obtained from Roche Diagnostics (Mannheim, Germany). A dye-binding protein assay kit is from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidene difluoride membrane and chemiluminescence detection kit are from Amercham Pharmacia Biotech. (Piscataway, NJ, USA).

2.2.2. Preparation of 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol fortified ginseng extract

The 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol fortified ginseng extract (GFGE) used in this study were prepared from Korean ginseng (*Panax ginseng* C.A. Meyer). The roots of 4-year old Korean ginseng were purchased in Poonggi Ginseng Nonghyup (Poonggi, Korea) and ginseng extract was manufactured by water extraction method by the Vitrosys Inc. (Poonggi, Korea). GFGE was standardized 8.0% of GPD and manufactured through enzymatic biotransformation by Cytolase PCL5 (DSM, Delft, Netherlands). Reaction mixture (pH 4.3) containing 7° Brix of ginseng extract was incubated for 84 h in 57℃. And then, the mixture (pH 6.5) was heated for 5 min in 95℃. After the reaction mixture was centrifuged, the pellet was dissolved in ethanol. The ethanol eluated solution was evaporated, and lyophilized to make powders. GFGE was analyzed using high-performance liquid chromatography (600S controller; Waters, Milford, MA, USA). After taken above procedures, GFGE contained mainly GPD: 8.2%, F2: 7.5%, and other minor ginsenosides, whereas there are mainly Rb1: 2.5%, Rc: 2.2%, Rb2: 1%, Rd: 0.8%, and other minor ginsenosides in raw white ginseng extract.

2.2.3. Animals

NC/Nga mice (female, 5-week-old) were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under specific pathogen-free

conditions at 22 ± 2 ℃ and 12-h light-dark cycle. All experimental procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Research Council (Washington DC, USA). The protocols for experiments were approved by Institutional Animal Care and Use Committee of Seoul National University (Seoul, Korea).

After 2 weeks of acclimation, mice were divided into 4 groups (n=10-11 per group). Seven-week-old female mice were orally administrated with GFGE (50-100 mg/kg/day, 150 μl/mouse/day) or vehicle (150 μl/mouse/day) for 28 d and further administered for 24 d together with topical application of DFE extract. Tacrolimus (0.1% Protopic® Ointment, Astellas Pharma)(100 μg/mouse/day) was applied topically onto shaved dorsal surface after 7 d of DFE application. The design of the study is summarized in Fig. 1.

2.2.4 Induction of AD-like symptoms

To induce AD-like symptoms in mice, DFE was applied topically. In detail, mice were anesthetized with isoflurane (Hana Pharm) and their hair on the back was removed using electric clipper (Daito Thrive, Tokyo, Japan) and cream (Reckitt Benckiser). For the disruption of skin barrier, 150 μl of 4% sodium dodecyl sulfate (Sigma) was topically applied on the surface.

Three hours later, shaved dorsal surfaces of mice were applied with 100 mg of Biostir cream (Biostir), which contains DFE twice a week for 4 weeks.

2.2.5. Assessment of dermatitis score

The skin lesions of mouse under anesthesia using 2% isoflurane were pictured once a week by digital camera (Canon SX40 HS, Canon, Tokyo, Japan). The dermatitis score was measured once a week according to a slight modification of the criteria described previously [18]. The dermatitis score was measured once a week. A total clinical score of dermatitis severity was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of four symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness).

2.2.6. Measurement of ear thickness and scratching time

After DFE treatment, the ears of mice became swollen, so I could confirm the severity of AD by measuring the ear thickness. During the 24-day DFE treatment, ear thickness was measured and recorded three times per week using a vernier caliper (Mitutoyo, Kanagawa, Japan). Scratching time was observed for 20 min per mouse once a week for the evaluation of scratching incidence.

2.2.7. Histopathological examination

The dorsal skin lesion of each mouse were fixed with 10 % neutral-buffered formalin, embedded in paraffin, and 4-μm-thick sections were cut and transferred onto slides. Deparaffinized skin sections were stained with Congo red or toluidine blue, to count the number of eosinophils and masts cells, respectively. Tissue sections were examined using an Olympus AX70 light microscope (Olympus, Tokyo, Japan) to count the number of eosinophils and mast cells per 0.025 mm^2 of skin at 400× magnification. The number of eosinophils and mast cells per mm^2 was calculated and presented in the RESULT.

2.2.8. Cytokine production in cultured splenocytes

To measure the cytokine production in cultured splenocytes, the spleen was aseptically removed from each animal. Red blood cells (RBC) were lysed using RBC lysis buffer (Qiagen), and the resulting lysate was centrifugated at 1500 × g for 10 min at a temperature of 4℃. The prepared splenocytes (5×10^6 cells/ml) in RPMI1640 media (Welgene) with 10% FBS (Sigma) were stimulated with plate-bound anti-CD3 (1 mg/ml) and anti-CD28 (3 mg/ml) monoclonal antibodies (mAbs) (BD Bioscience) at 37 ℃ under 5% CO_2 for 48 h. The supernatants were collected and stored at -80℃ until use. The levels of Th1 (IL-2, IL-12, and IFN-γ), Th2 (IL-4 and IL-5),

regulatory T cell (Treg) (IL-10), and pro-inflammatory (TNF-α and GM-CSF) cytokines were determined by multiplex Bio-Plex Pro™ assay (Bio-Rad Laboratory, Hercules, CA, USA), according to the manufacturer's instruction.

2.2.9. Statistical Analysis

The data were expressed as means ± standard error of the mean (SEM), and the significance of differences was determined using the Student's t-test. Probability values of $p < 0.05$, 0.01 and 0.001 were used as criterion for statistical significance.

Figure 1

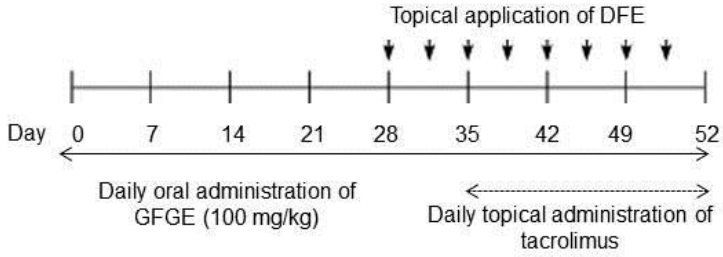


Figure 1. The entire schedule of study

2.3. Results

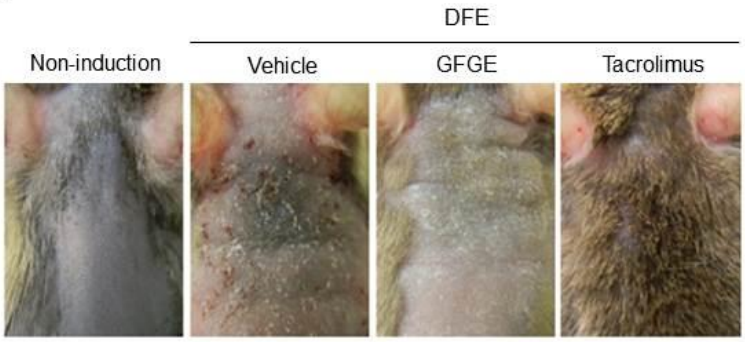
2.3.1. Oral administration of GFGE attenuated the clinical severity of DFE induced AD-like symptoms in NC/Nga mice

To investigate the effect of GFGE on DFE-induced AD-like symptoms in NC/Nga mice, we observed the phenotype of skin lesion and measured the dermatitis score. At the experiment day 52, the dorsal lesions of DFE-treated NC/Nga mice showed severe erythematous, erosive, and dried phenotype (Fig. 2A). However, the treatment with GFGE or medicinal ointment, tacrolimus, markedly attenuated the AD-like phenotype (Fig. 2A).

The dermatitis score was significantly increased in DFE and vehicle treated group at the experiment day 42 (5.36±0.31, $p < 0.001$) and 49 (6.36±0.41, $p < 0.001$), compared to non-induction group (1.10±0.18 and 0.90±0.18, respectively) (Fig. 2B). The treatment of GFGE (2.73±0.24, $p < 0.001$, and 2.82±0.54, $p < 0.001$) and tacrolimus (1.82±0.35, $p < 0.001$ and 1.18±0.26, $p < 0.001$) significantly lowered the dermatitis score increased by DFE (5.36±0.31 and 6.36±0.41) treatment at the experiment day 42 and 49, respectively (Fig. 2B).

Figure 2

A


DFE
Non-induction
Vehicle
GFGE
Tacrolimus

B

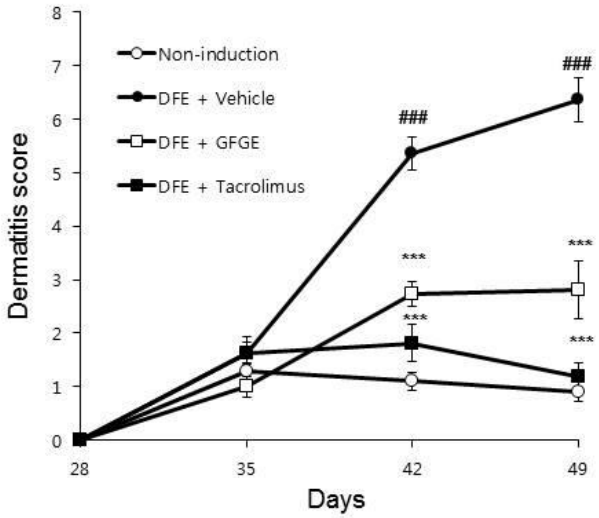

Non-induction
DFE + Vehicle
DFE + GFGE
DFE + Tacrolimus
Dermatitis score
Days
0
1
2
3
4
5
6
7
8
28
35
42
49
###
###
***
***
***
***

Figure 2. Effects of GFGE on DFE-induced clinical severity of AD-like symptoms in NC/Nga mice. *A*, The pictures of clinical features in NC/Nga mice were taken on day 52. *B*, Dermatitis scores were evaluated from day 28 to day 49 once a week. The data represents the mean ± SEM (n=10~11). $^{\#\#}$, significance at $p < 0.01$, $^{\#\#\#}$, $p < 0.001$, between DFE-treated AD mice and non-induction mice, ***,** $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, between GFGE or tacrolimus administered mice and DFE-treated AD mice (Student's t-test).

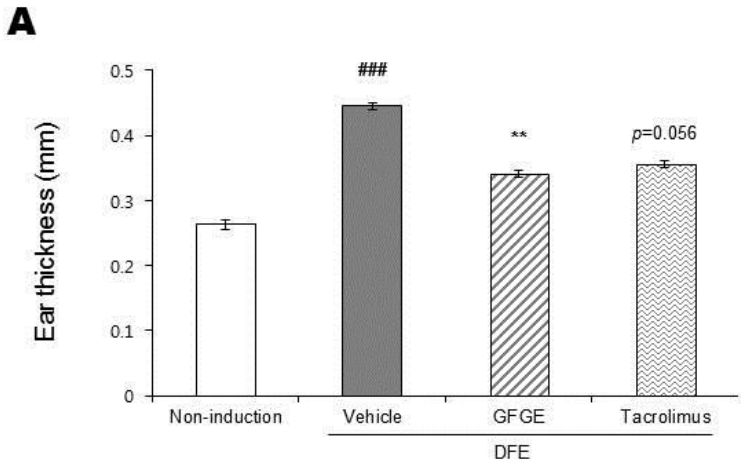
2.3.2. Oral administration of GFGE reduced DFE-induced ear swelling and scratching incidence in NC/Nga mice

The thickness of ears of all mice was measured at experiment day 52. It was found that the ears of DFE and vehicle treated mice become significantly thick (0.45±0.01 mm, $p < 0.001$) compared to non-induction group (0.26±0.01 mm) (Fig. 3A). On the other hand, the ears of DFE and GFGE treated mice were significantly less thick (0.34±0.00 mm, $p < 0.01$) compared to those of DFE and vehicle treated mice (0.45±0.01 mm) (Fig. 3A). There was no difference on the ear thickness of DFE and tacrolimus treated mice (0.36±0.01 mm, $p = 0.056$) and DFE and vehicle treated mice (Fig. 3A). As a result, GFGE markedly reduced the ear swelling induced by DFE, and the effect of which was better than tacrolimus.

Intensive pruritus, the hallmark of AD, precipitate extensive scratching [19]. Thus, the scratching time of NC/Nga mice was measured once a week. At experiment day 49, the scratching behavior was explosively increased in DFE and vehicle treated mice (273.64±40.99 sec, $p < 0.001$) compared to non-induction group (128.70±22.68 sec), whereas the treatment of GFGE (107.18±16.28 sec, $p < 0.01$) or tacrolimus (81.73±12.00 sec, $p < 0.001$) reduced the scratching incidence increased by DFE treatment (273.64±40.99 sec) (Fig. 3B). On the other hand, at experiment day 42, the

treatment of tacrolimus (29.82±7.67, $p < 0.01$) decreased the scratching incidence of non-induction group (95.30±10.67 sec) (Fig. 3B).

Figure 3


A
Ear thickness (mm)
0.5
0.4
0.3
0.2
0.1
0
###
**
p=0.056
Non-induction
Vehicle
GFGE
Tacrolimus
DFE

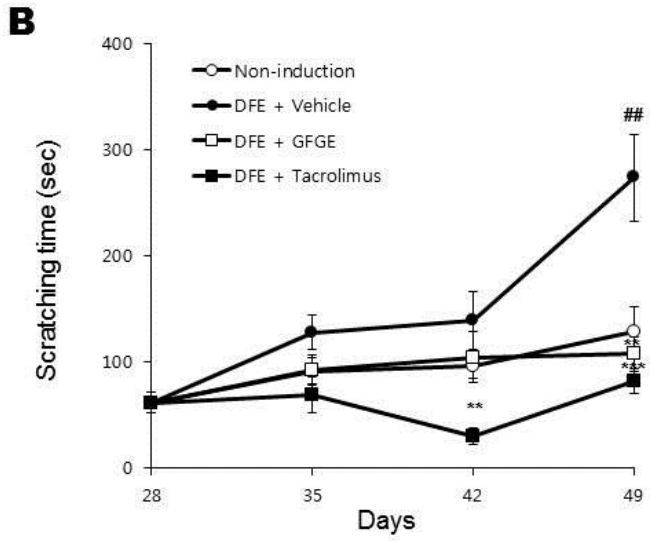

B
Scratching time (sec)
400
300
200
100
0
Non-induction
DFE + Vehicle
DFE + GFGE
DFE + Tacrolimus
##
**
**
**
28
35
42
49
Days

Figure 3. Effects of GFGE on DFE-induced ear thickness and scratching incidence in NC/Nga mice.

A, Ear thickness in NC/Nga mice were measured on day 52. *B*, scratching time were evaluated from day 28 to day 49 once a week. The data represents the mean ± SEM (n=10~11). $^{\#\#}$, significance at $p < 0.01$, $^{\#\#\#}$, $p < 0.001$, between DFE-treated AD mice and non-induction mice**, *,** $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, between GFGE or tacrolimus administered mice and DFE-treated AD mice (Student's t-test).

2.3.3. Oral administration of GFGE inhibited DFE-induced infiltration of eosinophils and mast cells in skin lesion of NC/Nga mice

Congo red or toluidine blue was used to stain eosinophils and mast cells infiltrated in the skin of NC/Nga mice, respectively (Fig. 4A). The number of Congo red-stained eosinophils in skin lesion of DFE and vehicle treated mice (974.55 cells±54.81 per mm^2, $p < 0.001$) was significantly increased compared to non-induction group (44.00±12.58 cells per mm^2) (Fig.4A and B). On the other hand, the number of eosinophils in skin of DFE and GFGE (425.46±38.24 cells per mm^2, $p < 0.001$) or DFE and tacrolimus (654.55±46.79 cells per mm^2, $p < 0.001$) treated mice was markedly decreased compared to DFE and vehicle treated mice (974.55±54.81 cells per mm^2) (Fig. 4A and B).

The number of toluidine blue-stained mast cells in skin lesion of DFE and vehicle treated mice (949.09±115.44 cells per mm^2, $p < 0.001$) was significantly increased compared to non-induction group (348.00±40.46 cells per mm^2) (Fig. 4A and C). GFGE treatment (625.46±48.62 cells per mm^2, $p < 0.001$) significantly reduced the number of infiltrated mast cells in skin lesion of DFE treated mice (949.09±115.44 cells per mm^2) (Fig. 4A and C). Tacrolimus treatment (912.73±61.93 cells per mm^2) did not alter the number of infiltrated mast cells in skin lesion of DFE treated mice (949.09±115.44 cells per mm^2) (Fig. 4A and C).

Figure 4

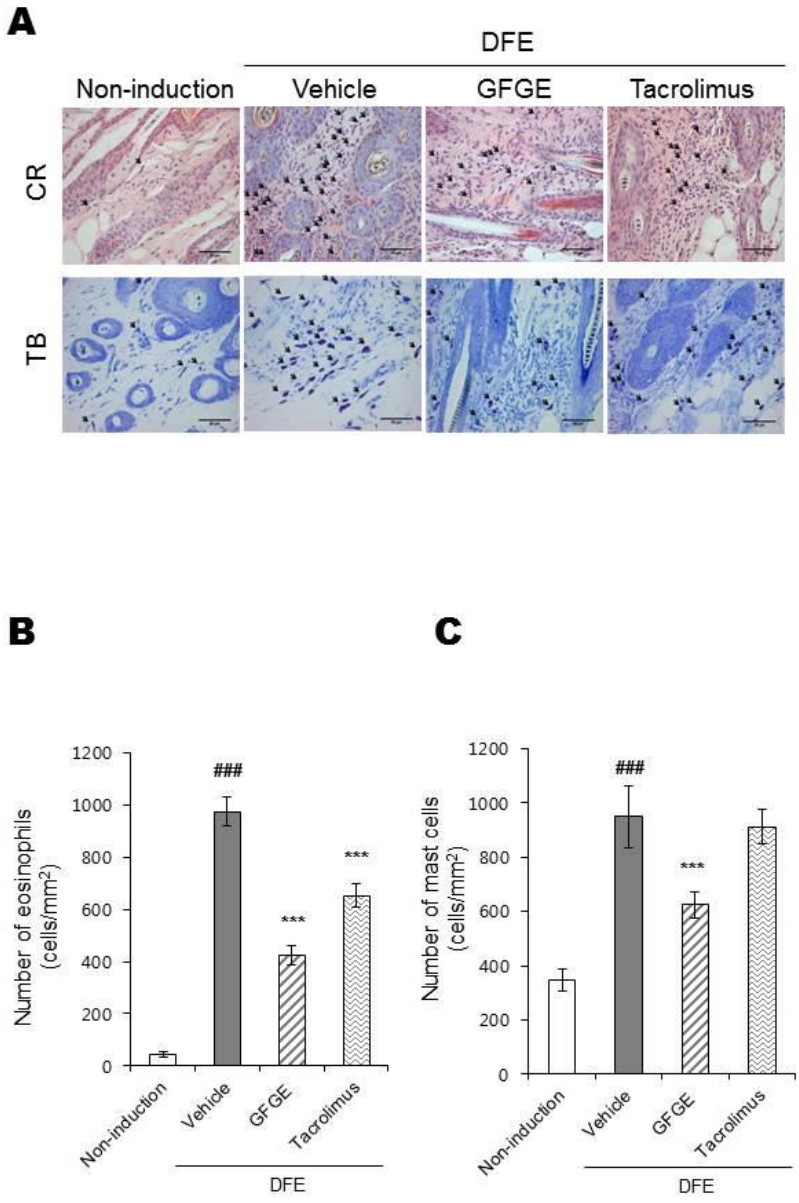

A
DFE
Non-induction
Vehicle
GFGE
Tacrolimus
CR
TB
B
Number of eosinophils (cells/mm²)
1200
1000
800
600
400
200
0
###
***
***
Non-induction
Vehicle
GFGE
Tacrolimus
DFE
C
Number of mast cells (cells/mm²)
1200
1000
800
600
400
200
0
###
***
Non-induction
Vehicle
GFGE
Tacrolimus
DFE

Figure 4. Effects of GFGE on DFE-induced infiltration of eosinophils and mast cells in skin lesion.

A, Representative histological features of eosinophils (Congo red (CR) staining) and mast cells (toluidine blue (TB) staining) in skin lesion. Arrows indicate the CR-stained eosinophils and the TB-stained mast cells. The number of cells was counted under a microscope at 400× magnification. *B,* The number of eosinophils in the 1 mm^2 of skin lesion. *C,* The number of mast cells in the 1 mm^2 of skin lesion. The data represents the mean ± SEM (n=10~11). $^{\#\#}$, significance at $p < 0.01$, $^{\#\#\#}$, $p < 0.001$, between DFE-treated AD mice and non-induction mice, ***,** $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, between GFGE or tacrolimus administered mice and DFE-treated AD mice (Student's t-test). Scale bar=50 μm.

2.3.4. Splenocytes of DFE and GFGE treated NC/Nga mice produced less cytokines compared to those of DFE and vehicle-treated mice

To examine the effect of GFGE in the production of cytokines, splenocytes derived from NC/Nga mice were stimulated with anti-CD3 and anti-CD28 mAbs. The level of Th1 cytokines (IL-2, IL-12, and IFN-γ), Th2 cytokines (IL-4 and IL-5), Treg cytokine (IL-10), and pro-inflammatory cytokines (TNF-α and GM-CSF) in splenocyte supernatant was measured. The level of IL-12, IL-4, IL-5, IL-10, TNF-α, and GM-CSF was significantly increased in DFE and vehicle treated mice (IL-12, 235%, $p < 0.001$; IL-4, 294%, $p < 0.001$; IL-5, 338%, $p < 0.001$; IL-10, 212%, $p < 0.001$; TNF-α, 164%, $p < 0.01$; GM-CSF, 163%, $p < 0.01$) compared to non-induction group (Fig. 5A and B). However, the level of IL-2 and IFN-γ was not significantly different in DFE and vehicle treated mice (IL-2, 102% and IFN-γ, 110%) compared to non-induction group (Fig. 5A and B). Splenocytes derived from NC/Nga mice treated with DFE and GFGE produced significantly less level of various cytokines (IL-2, 55%, $p < 0.01$; IL-12, 81%, $p < 0.001$; IFN-γ, 52%, $p < 0.001$; IL-4, 88%, $p < 0.001$; IL-5, 63%, $p < 0.001$; IL-10, 110%, $p < 0.001$; TNF-α, 80%, $p < 0.001$; GM-CSF, 63%, $p < 0.001$), compared to those treated with DFE and vehicle (Fig. 5A and B). Splenocytes derived from NC/Nga mice treated with DFE and tacrolimus also produced significantly less level of various cytokines (IL-2, 31%, $p <$

0.001; IL-12, 88%, $p < 0.001$; IFN-γ, 20%, $p < 0.001$; IL-4, 101%, $p < 0.001$; IL-5, 54%, $p < 0.001$; IL-10, 92%, $p < 0.001$; TNF-α, 53%, $p < 0.001$; GM-CSF, 32%, $p < 0.001$) compared to those treated with DFE and vehicle (Fig. 5A and B).

Figure 5

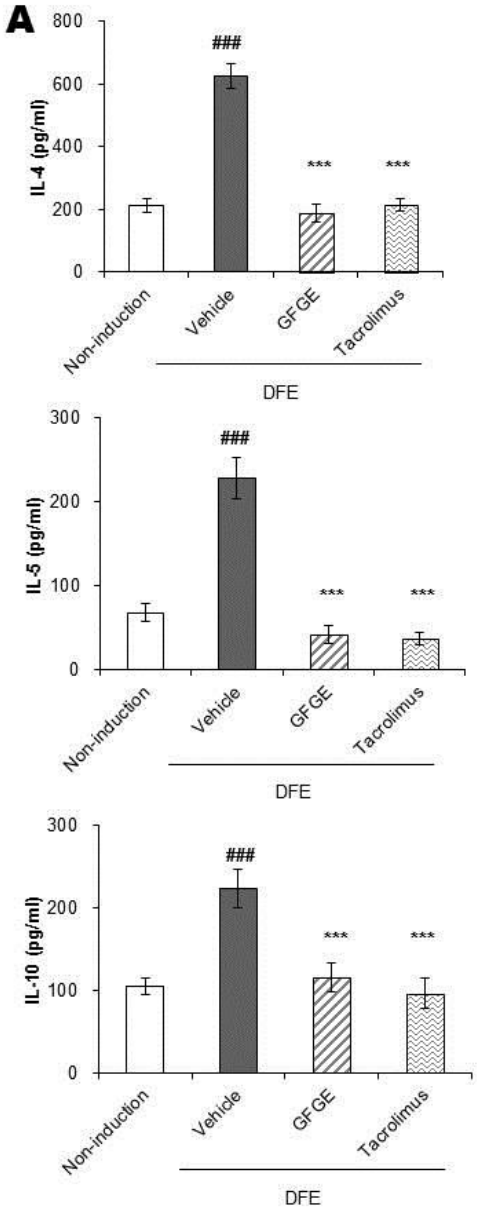

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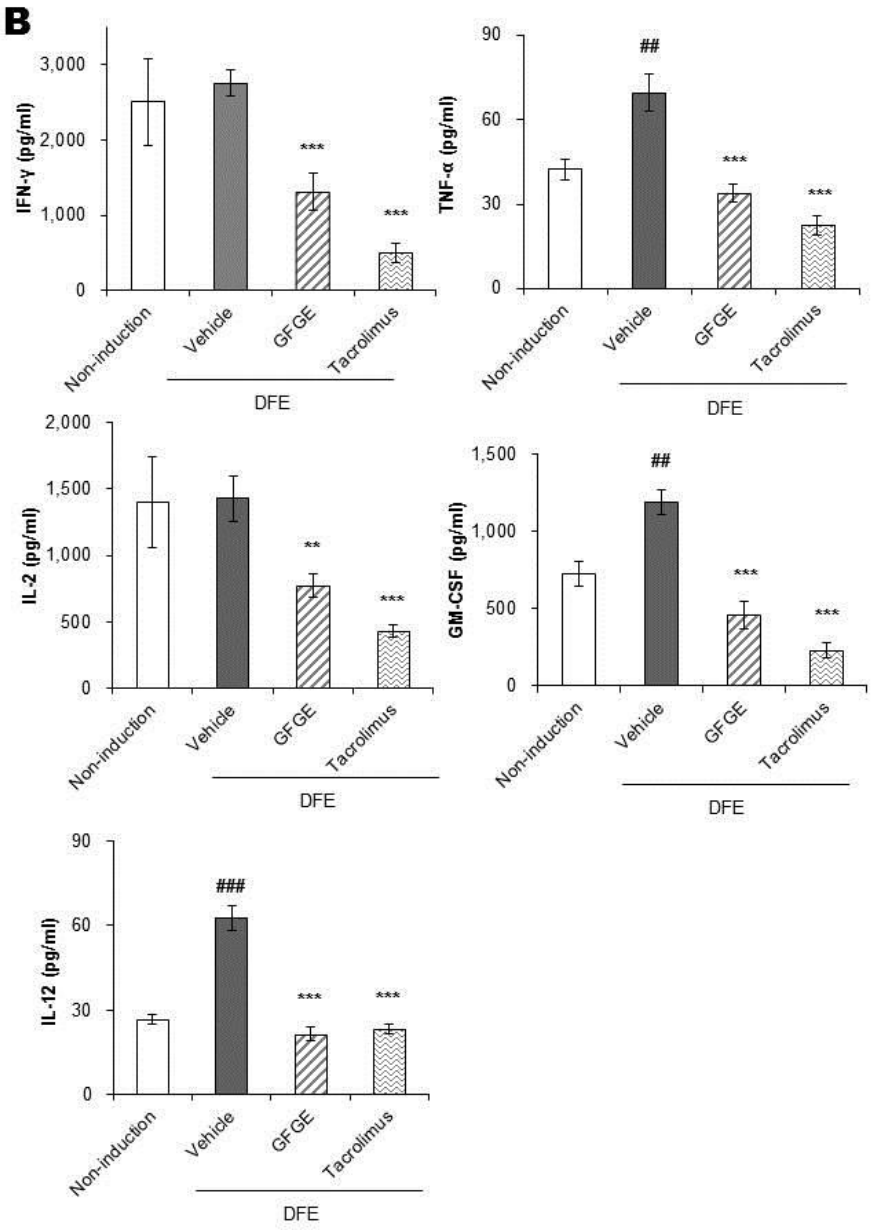
Figure 5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Vehicle
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Tacrolimus
DFE

Figure 5. The effects of GFGE on the level of cytokines in cultured splenocytes.

The production of Th1, Th2, Treg and pro-inflammatory cytokines in splenocytes derived from NC/Nga mice. The level of cytokines was analyzed in splenocytes stimulated with anti-CD3 and anti-CD28 in 10% FBS RPMI1640 for 48 h. The data represents the mean ± SEM (n=8). $^{\#\#}$, significance at $p < 0.01$, $^{\#\#\#}$, $p < 0.001$, between DFE-treated AD mice and non-induction mice, *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, between DFE and GFGE or tacrolimus treated mice, and DFE and vehicle-treated mice (Student's t-test).

2.4. Discussion

GPD was suggested as a candidate therapeutic agent for allergy treatment [9]. In this study, I found that GFGE, which contains 8.2% of GPD, attenuate the DFE-induced AD-like symptoms in NC/Nga mice. GFGE treatment significantly decreased the DFE-induced severity of skin lesion, increase in dermatitis score, ear swelling, and scratching incidence in NC/Nga mice. GFGE treatment significantly decreased the DFE-induced infiltration of eosinophils and mast cells in skin lesion of NC/Nga mice. I also observed that splenocytes of DFE and GFGE treated NC/Nga mice produced less cytokines including IL-2, IL-12, IFN-γ, TNF-α, GM-CSF, IL-4, IL-5, and IL-10 compared to those of DFE and vehicle-treated NC/Nga mice.

The recruitment of eosinophils and mast cells during allergic disease is associated expression of cytokines [20, 21]. For example, IL-5 has been suggested to play an important role for the production of eosinophils [22]. IL-5 deficient mice showed inability to develop eosinophilia under allergen exposure [23]. Dent et al. demonstrated that IL-5 produces eosinophilia and induces the full pathway of eosinophil differentiation [24]. GM-CSF has been also reported to generate the eosinophils in bone marrow [25]. On the other hand, IL-4 is known to activate the mast cells [26]. Since I found that splenocytes of DFE and GFGE treated NC/Nga mice produce less IL-5, GM-CSF, and IL-4 compared to those of DFE and vehicle-treated NC/Nga mice

(Table 1), the effect of GEGE on the less production of IL-5, GM-CSF, and IL-4 may be related with less infiltration of eosinophils and mast cells in AD lesions of NC/Nga mice.

In skin lesion of AD, there is a significant increase in the number of cells expressing Th2 and Treg cytokines [27, 28]. Transgenic mice which overproduce Th2 cytokines, such as IL-4 and IL-5 have been reported to contribute to develop atopic dermatitis, spontaneously [4]. The percentage and absolute numbers of Treg cells producing IL-10 were significantly elevated in AD patient [28]. And predominance of Th1 cytokines, especially IFN-γ, determines severity of the disease [29]. Chen et al. suggested that IL-2, IFN-γ , and IL-12 producing cells increased in skin lesion, particularly mice with chronic AD [7]. It has been reported that scratching on AD skin lesion causes mechanical injury, resulting in increased production of pro-inflammatory cytokines such as IL-1, TNF-α, and GM-CSF [8]. I found that GFGE inhibited the DFE-mediated production of Th1 cytokines (IL-12), Th2 cytokines (IL-4, IL-5), Treg cytokine (IL-10), and pro-inflammatory cytokines (TNF-α, GM-CSF). In this study, IL-2 and IFN-γ were not significantly elevated by DFE administration compared to non-induction group, this phenomenon could be explained that decreased Th1 response in early phase of AD less increased at end of experiment.

Although several AD therapeutic drugs such as steroids and anti-histamine are commonly available, the effects are moderate and cause some adverse effects including hypertension, osteoporosis, iatrogenic Cushing's disease, dizziness, and blurred vision [1]. Recently, calcineurin inhibitors, such as tacrolimus and pimecrolimus, have been topically used and considered quite safe [30-32], however, prolonged application of these drugs is also reported to cause skin cancer [33]. Therefore, the development of alternative therapeutics is required for long-term management of AD.

In conclusion, these results demonstrated that oral administration of GFGE in NC/Nga mice reduced DFE-induced AD-like symptoms including AD-like phenotypes, dermatitis score, ear swelling, scratching behavior, and infiltration of eosinophils and mast cells in skin lesion. Splenocytes derived from NC/Nga mice treated with DFE and GFGE produced less cytokines including IL-2, IL-12, IFN-γ, TNF-α, GM-CSF, IL-4, IL-5, and IL-10, compared to those treated with DFE and vehicle. These observations suggest that early management of GFGE might prevent the development of AD and be potent alternative therapeutic.

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Chapter 3.

20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol fortified ginseng extract improves the symptoms of atopic dermatitis

Abstract

Atopic dermatitis (AD) is chronically relapsing inflammatory skin disease, accompanied by severe itching and dryness. AD is often seen in infants and children; its incidence is increasing globally. Therefore, AD is referred to a major public health problem worldwide. AD is thought to trigger other allergic diseases through percutaneous sensitization of allergens. And AD in infancy and childhood can be extended to adulthood depending on genetic predisposition, and circumstance environmental conditions. Because many therapeutic drugs have side effects, especially chronic usage, development of complementary, alternative therapeutic approaches are essential. In this study, 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD) fortified ginseng extract (GFGE) displayed to inhibit skin inflammation, itching, and level of Th1/Th2 (IFN-γ, IL-5 and IL-13) cytokines, and macrophage derived chemokine (MDC). And these effects are accompanied to reduction of the inflammatory cells infiltration and enlargement of draining lymph node. These data suggest the oral administration of GFGE can improve the symptoms of prior developed AD, and it can be effective as complementary and alternative therapeutic agent.

Key words: *Atopic dermatitis; Ginseng, 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD); Immunomodulation; Dermatophagoides farinae;*

NC/Nga; Th 1/2 cytokine, macrophage-derived chemokine (MDC), brachial lymph node

3.1. Introduction

Atopic dermatitis (AD) is chronically relapsing inflammatory skin disease, accompanied by severe itching and dryness [1]. Pruritus make the patient to scratch, it destroy the skin barrier and exacerbate the AD [2] . AD is often seen in infants and children, its incidence is increasing. Many epidemiological studies have demonstrated that AD is one of major public health problem worldwide [3]. AD is developed by complicated interactions with genetic predisposition and environmental factors [4]. Skin lesions in AD patient show the epidermal and dermal hypertrophy, and infiltration of inflammatory cells, including eosinophils, mast cells and macrophages.

Although generalized Th2-polarized immune response is closely linked to AD, the skin inflammation shows biphasic responses with an initial Th2 phase and chronic Th0/Th1 profile, with an accumulation of IFN-γ producing cells [5]. Scratching induce mechanical injury in skin, resulting in increased proinflammtory chemokine such as macrophage-derived chemokine (MDC) [6]. Several medicinal drugs have been used to treatment; however they have been reported to have some mild to severe side effects. Therefore, relevant studies have focused on complementary therapies based on alternative medicine.

Ginseng (*Panax ginseng* C.A. Meyer) is one of the most popular medicinal herbs, which has been taken for a long time, especially Asian

countries. Ginseng contains various pharmaceutical components such as ginsenosides, polyacetylenes, polyphenolic compounds, and acidic polysaccharides, most of all, ginsenosides have been considered to most effective components [7]. In ginseng root, there are five major ginsenosides (Rb1, Rb2, Rc, Re, and Rg1), which consist of more than 80% of total ginsenosides [8]. Recently, many studies have focused on pharmaceutical effects of the minor ginsenosides, such as Rg3, Rh2, F2, and 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (GPD, previously named as compound K), because their activities are shown to be superior than those of major ginsenosides. Among them, GPD is the main metabolite of protopanaxadiol type ginseng saponins in intestine after oral administration and also is the major form of protopanaxadiol saponins absorbed to the body [9]. However, GPD is rare in raw ginseng; because of this, many studies have aimed to convert from major ginsenosides to GPD. The GPD fortified ginseng extract (GFGE) through unique process has developed, and GPD is contained above 8 % in GFGE. And I investigated its anti-allergic effect on atopic dermatitis model in NC/Nga mouse.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol fortified ginseng extracts (GFGE) is obtained by CJ Food R&D (CJ CheilJedang, Seoul, Korea). AD Biostir cream, *Dermatophagoides farinae* extract (DFE), is obtained from Biostir (Hiroshima, Japan). Tacrolimus (0.1% Protopic® Ointment) is from Astellas Pharma (Tokyo, Japan). Anti-CD3 mAb, and anti-CD28 mAb are from BD Bioscience (San Jose, CA, USA). The ELISA kits of IL-13, IFN-γ, and MDC (R&D Systems, Minneapolis, MN, USA), and IL-5 (Biolegend, San Diego, CA, USA) are purchased.

3.2.2. Preparation of 20-O-β-D-glucopyranosyl-2-(S)-protopanaxadiol fortified ginseng extract

The GFGE was prepared by CJ Food R&D (CJ CheilJedang, Seoul), previously reported. Briefly, The GFGE was prepared from the roots of 4-year Korean ginseng (*Panax ginseng* C.A. Meyer) (Poonggi Ginseng Nonghyup, Poonggi, Korea). Raw white ginseng extract was manufactured by water extraction method by the Vitrosys Inc. (Poonggi, Korea). GFGE was manufactured from raw white ginseng extract through enzymatic biotransformation by Cytolase PCL5 (DSM, Delft, Netherlands). GFGE was analyzed by high-performance liquid chromatography (600S controller;

Waters, Milford, MA, USA). After taken above procedures, GFGE contained mainly GPD: 8.2%, F2: 7.5%, and other minor ginsenosides, whereas there are mainly Rb1: 2.5%, Rc: 2.2%, Rb2: 1%, Rd: 0.8%, and other minor ginsenosides in raw white ginseng extract.

3.2.3. Animals

NC/Nga mice (female, 5-week-old) were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under specific pathogen-free conditions at 22 ± 2℃ and 12-h light-dark cycle. All experimental procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals (ILAR, NRC, Washington D.C., USA) and the protocols were approved by Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea.

After 2 weeks acclimation, mice were divided into 6 groups (*n*=8/group). Seven-week-old female mice were induced atopic-like symptom with *Dermatophagoides farinae* body extract (DFE) except non-induction group for 9 weeks. 3 weeks after first administration of DFE, all mice were orally administrated with GFGE (50 or 100 mg/kg/day, 150 μl/mouse/day) or vehicle (150 μl/mouse/day) once a day for 42 days. Tacrolimus (0.1% Protopic® Ointment, Astellas Pharma)(100 μg/mouse/day) was applied topically onto shaved dorsal surface once a day

for 42 day. The design of the study is summarized in Fig. 1.

3.2.4 Induction of AD-like symptoms

To induce AD-like symptoms in mice, DFE was applied topically. In detail, mice were anesthetized with isoflurane (Hana Pharm) and their hair on the back was removed using electric clipper (Daito Thrive, Tokyo, Japan) and cream (Reckitt Benckiser). For the disruption of skin barrier, 150 μl of 4% sodium dodecyl sulfate (Sigma) was topically applied on the surface. Three hours later, shaved dorsal surfaces of mice were applied with 100 mg of Biostir cream (Biostir), which contains DFE twice a week for 4 weeks.

3.2.5. Assessment of dermatitis score

The skin lesions of mouse under anesthesia using 2% isoflurane were pictured once a week by digital camera (Canon SX40 HS, Canon, Tokyo, Japan). The dermatitis score was measured once a week according to a slight modification of the criteria described previously [10]. The dermatitis score was measured once a week. A total clinical score of dermatitis severity was defined as the sum of the individual scores graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of four symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness).

3.2.6. Measurement of scratching time and skin thickness

At day 42, scratching time was observed for 20 min per mouse for the evaluation of scratching incidence, And the skin thickness were measured using a vernier caliper (Mitutoyo, Kanagawa, Japan).

3.2.7. Histopathological examination

The dorsal skin lesion of each mouse were fixed with 10 % neutral-buffered formalin, embedded in paraffin, and 4-μm-thick sections were cut and transferred onto slides. Deparaffinized skin sections were stained with hematoxylind & eosin (H&E), Congo red (CR) or toluidine blue (TB), to assess the epidermal and dermal hypertrophy, and to count the number of eosinophils and masts cells, respectively. Tissue sections were examined using an Olympus AX70 light microscope (Olympus, Tokyo, Japan) under 100× (H&E staining) and 400× magnification(CR, TB staining). To count the number of eosinophils and mast cells per 0.025 mm^2 of skin, and the number of eosinophils and mast cells per mm^2 was calculated and presented in the RESULT.

3.2.8. Assessment of swelling of brachial lymph nodes

To assess for grade of swelling in draining lymph nodes, brachial lymph nodes were removed from each mouse. The weights and the major

axis lengths of lymph nodes were measured by an electronic scale and a vernier caliper (Mitutoyo).

3.2.9. Analysis for the levels of Th1/Th2 cytokine in cultured splenocytes and MDC in serum

To measure the Th1/Th2 cytokine production in cultured splenocytes, the spleen was aseptically removed from each animal. Red blood cells (RBC) were lysed using RBC lysis buffer (Qiagen), and the resulting lysate was centrifugated at 1500 × g for 10 min at a temperature of 4℃. The prepared splenocytes (5×10^6 cells/ml) in RPMI1640 media (Welgene) with 10% FBS (Sigma) were stimulated with plate-bound anti-CD3 (1 mg/ml) and anti-CD28 (3 mg/ml) monoclonal antibodies (mAbs) (BD Bioscience) at 37 ℃ under 5% CO_2 for 48 h. The supernatants were collected and stored at -80℃ until use. To assess the level of MDC in serum, whole blood was obtained from each mouse by cardiac puncture under deep anesthesia at the end of experiment. The serum was collected and stored at -80℃ until use. The levels of IL-5, IL-13, IFN-γ, and MDC were determined by ELISA kits (Biolegends and R&D Systems) according to the manufacturer's instructions.

3.2.10. Statistical Analysis

The data were expressed as means ± standard error of the mean

(SEM). One-way analysis of variance (ANOVA) was used for comparisons in the experiments. When ANOVA indicated statistical significance, Duncan's multiple range test was used to determine which means were significantly different. A probability value of $p < 0.05$ was used as criterion for statistical significance.

Figure 1

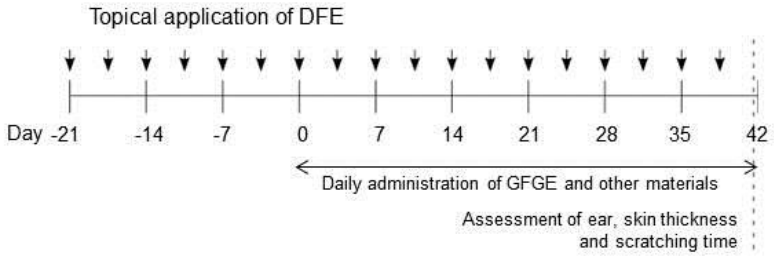


Figure 1. The entire schedule of study

The day after completion of the clinical assessment, all mice were sacrificed and spleen, and skin were removed for Th1/Th2 cytokine analysis, and histopathological examination

3.3. Results

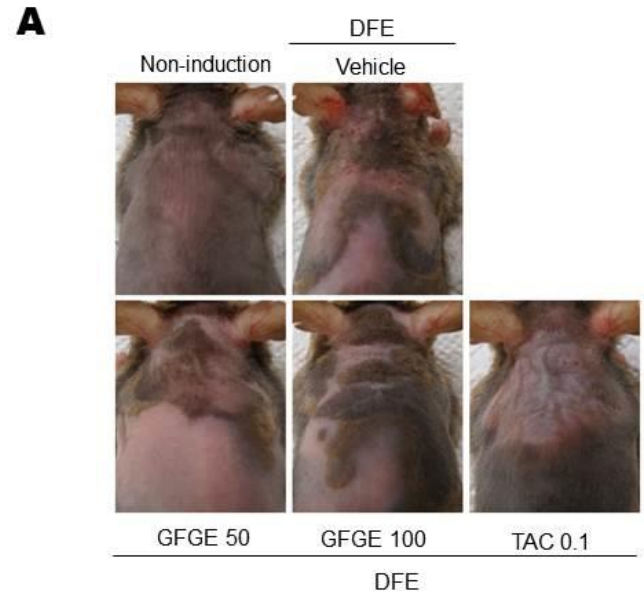
3.3.1. Oral administration of GFGE attenuated the clinical severity of DFE induced AD-like symptoms in NC/Nga mice

To investigate the clinical effect of GFGE on AD in NC/Nga mice, I assessed the dermatitis severity. On day 42, severe dermatitis was observed in DFE with vehicle administered mice. Their dorsal lesions showed severe xerosis, erythematous, and excoriated. Treatment with GFGE, calcineurin inhibitor, tacrolimus, and red ginseng extract markedly improved the dermatitis severity (Fig. 2A). The dermatitis scores were significantly increased in DFE with vehicle treated group, compared to non-induction group every two weeks from day 0 to day 42. However, the administration with GFGE 50 mg/kg (GFGE 50), GFGE 100 mg/kg (GFGE 100), and tacrolimus 100 μg (TAC 0.1) significantly lowered dermatitis score (Fig. 2B). As shown in Fig. 2B, the drug, TAC 0.1, had most effective action, the mice administered with GFGE 50 were shown the slightly better condition than them administered with GFGE 100.

The scratching incidence, thickness of dorsal skin of all mice was measured at day 42. The scratching behavior was explosively increased by DFE application (221.78±27.24 sec) compared to non-induction group (31.62±11.99 sec). Whereas GFGE 50 (28.55±6.57 sec), GFGE 100 (63.60±17.66 sec), and TAC 0.1 (29.08±3.63 sec) administration reduced the

scratching incidence compared to DFE with vehicle administered mice (Fig. 3A).

Figure 2


A
DFE
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

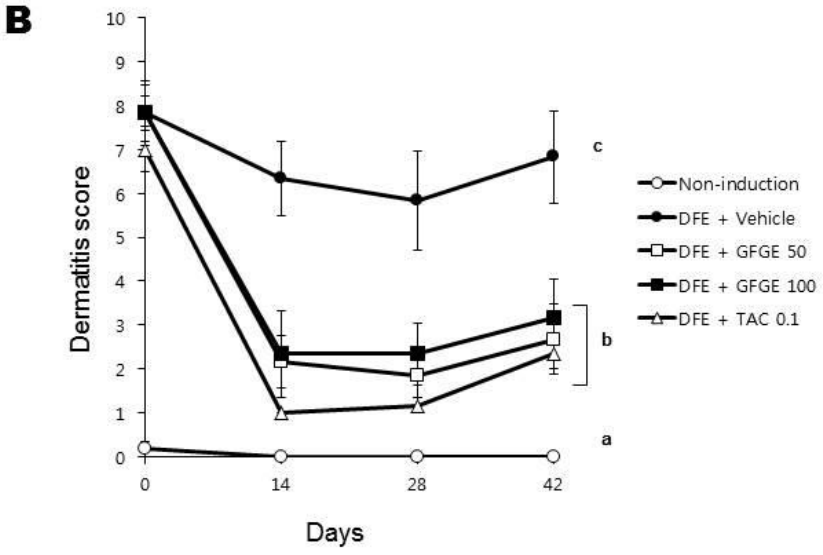

B
Dermatitis score
Days
0
14
28
42
c
b
a
Non-induction
DFE + Vehicle
DFE + GFGE 50
DFE + GFGE 100
DFE + TAC 0.1

Figure 2

C

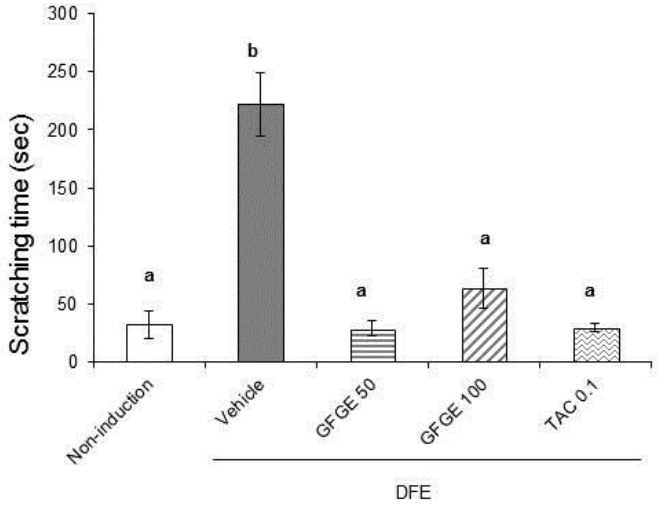
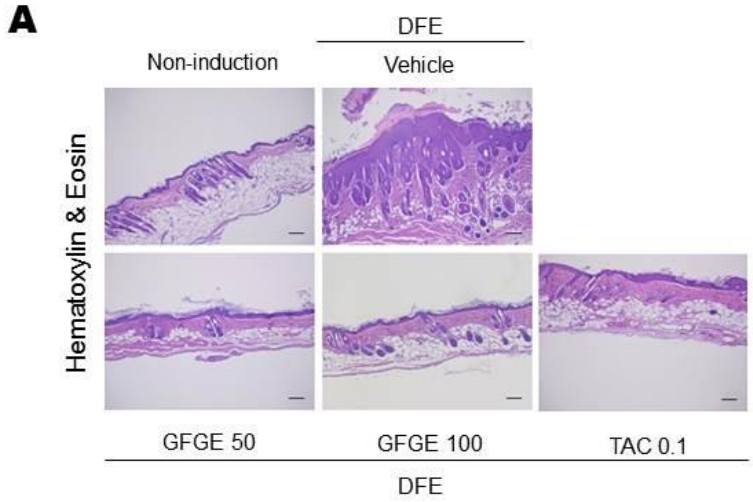

Scratching time (sec)
300
250
200
150
100
50
0
a
b
a
a
a
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

Figure 2. Effects of GFGE on the clinical severity of AD in NC/Nga mice. *A*, Clinical features in NC/Nga mice were taken on day 42. *B*, Dermatitis scores were evaluated from day 0 to day 42 every two weeks. *C*, Scratching time were evaluated at day 42. Data represents the mean ± SEM (n=7~8). Means with letters (a-c) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. GFGE 50, GFGE 50 mg/kg; GFGE 100, GFGE 100 mg/kg; TAC 0.1, tacrolimus 100 μg

3.3.2. Oral administration of GFGE reduced DFE-induced skin hypertrophy in NC/Nga mice

According to H&E staining histological assessment, the dorsal skin a hypertrophied by DFE administration compared to non-induction group (Fig. 3A). The dorsal skins of DFE with vehicle administered mice become significantly thick (0.38±0.03 mm) compared to non-induction group (0.16±0.00 mm). On the other hand, the ears of DFE with GFGE 50, GFGE 100, and TAC 0.1 administered mice were less thick (0.30±0.02 mm, 0.30±0.01 mm, and 0.30±0.01 mm) (Fig. 3B) Overall, I found that DFE-induced AD symptoms were suppressed in DFE with GFGE administered mice.

Figure 3


A
DFE
Non-induction
Vehicle
Hematoxylin & Eosin
GFGE 50
GFGE 100
TAC 0.1
DFE

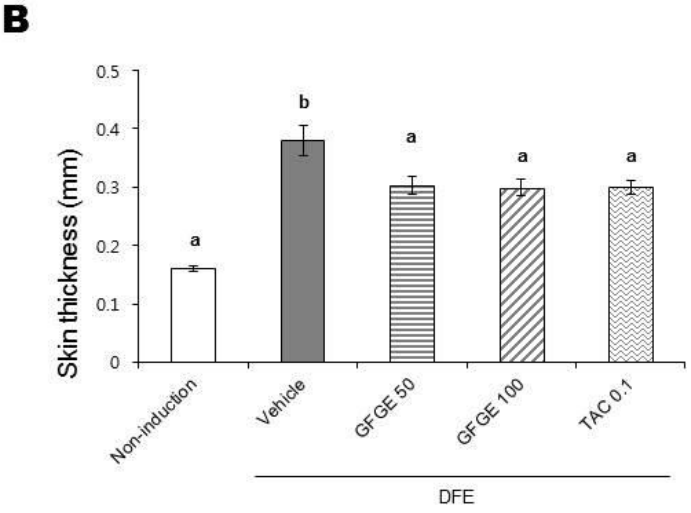

B
Skin thickness (mm)
0.5
0.4
0.3
0.2
0.1
0
a
b
a
a
a
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

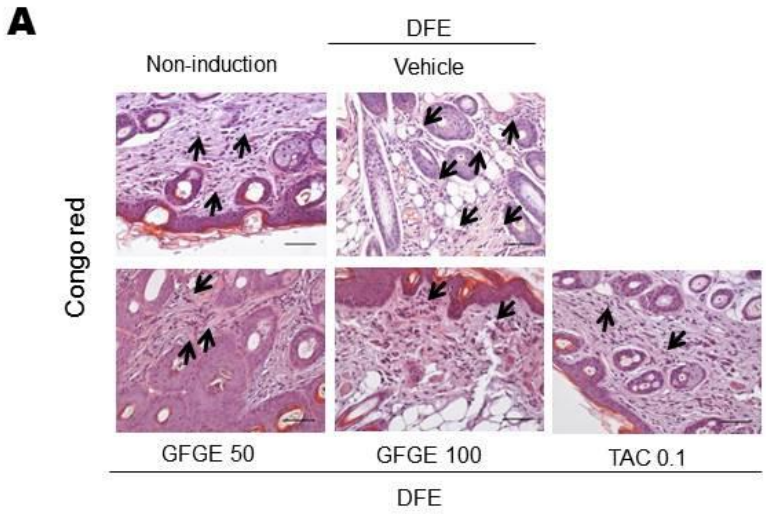
Figure 3. Effects of GFGE on skin hypertrophy in NC/Nga mice.

A, Representative histological features of hypertrophy (Hematoxylin & eosin staining) in skin lesion. 100× magnification. Scale bar: 50 μm. *B*, Dorsal skin thickness was evaluated at day 42 using vernier calipers. The data represents the mean ± SEM (n=7~8). Means with letters (a,b) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. GFGE 50, GFGE 50 mg/kg; GFGE 100, GFGE 100 mg/kg; TAC 0.1, tacrolimus 100 μg

3.3.3. Oral administration of GFGE inhibited DFE-induced infiltration of eosinophils in skin lesion of NC/Nga mice

Improvement of skin condition by GFGE was also confirmed by the analysis of Congo red stained section. DFE with vehicle administered mice exhibited a marked infiltration of eosinophils. However, administration of GFGE 50, GFGE 100, and TAC 0.1 reduced the infiltration of eosinophils (Fig. 4A). The number of eosinophils in skin lesion of DFE with vehicle administered mice were increased (200.00±29.21 cells per mm^2), compared to non-induction group (32.00±8.00 cells per mm^2). On the other hand, the cells were less infiltrated in GFGE 50, GFGE 100, and TAC 0.1 administered group (93.33±24.59 cells per mm^2; 86.67±6.67 cells per mm^2; 73.33±12.30 cells per mm^2) (Fig. 4B). DFE-induced infiltration of eosinophils on skin lesion was suppressed dose-dependent manner by GFGE administration

Figure 4


A
DFE
Non-induction
Vehicle
Congo red
GFGE 50
GFGE 100
TAC 0.1
DFE

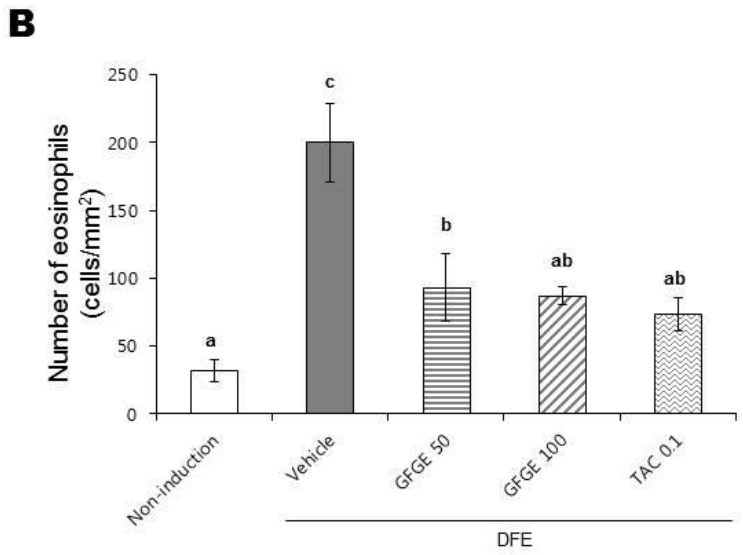

B
Number of eosinophils (cells/mm2)
250
200
150
100
50
0
a
c
b
ab
ab
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

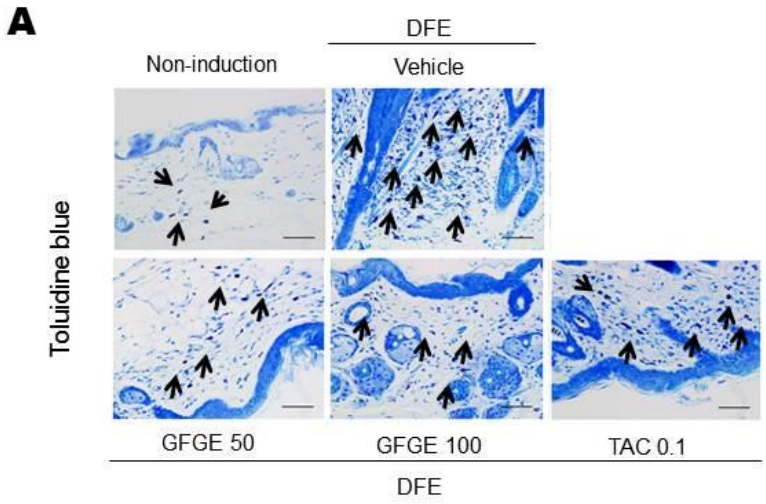
Figure 4. Effect of GFGE on accumulation of eosinophils in skin lesion.

A, Representative histological features of eosinophils (Congo red (CR) staining) in skin lesion. Arrows indicate the CR-stained eosinophils. The number of cells was counted under a microscope at 400× magnification. Scale bar: 50 μm. *B*, The number of eosinophils in the 1 mm^2 of skin lesion. The data represents the mean ± SEM (n=7~8). Means with letters (a-c) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. GFGE 50, GFGE 50 mg/kg; GFGE 100, GFGE 100 mg/kg; TAC 0.1, tacrolimus 100 μg

3.3.4. Oral administration of GFGE inhibited DFE-induced infiltration of mast cells in skin lesion of NC/Nga mice

Improvement of skin condition by GFGE was also confirmed by the analysis of toluidine blue stained section. DFE with vehicle administered mice exhibited a marked infiltration of mast cells. However, administration of GFGE 50, GFGE 100, and TAC 0.1 reduced the infiltration of mast cells (Fig. 5A). The number of mast cells in skin lesion of DFE with vehicle administered mice were increased (1413.33±108.16 cells per mm^2), compared to non-induction group (384.00±20.40 cells per mm^2). On the other hand, the cells were less infiltrated in GFGE 50, GFGE 100, and TAC 0.1 administered group (593.33±47.80 cells per mm^2; 673.33±61.46 cells per mm^2; and 860.00±81.81 cells per mm^2) (Fig. 5B). DFE-induced infiltration of mast cells on skin lesion was suppressed by GFGE administration.

Figure 5


A
DFE
Non-induction
Vehicle
Toluidine blue
GFGE 50
GFGE 100
TAC 0.1
DFE

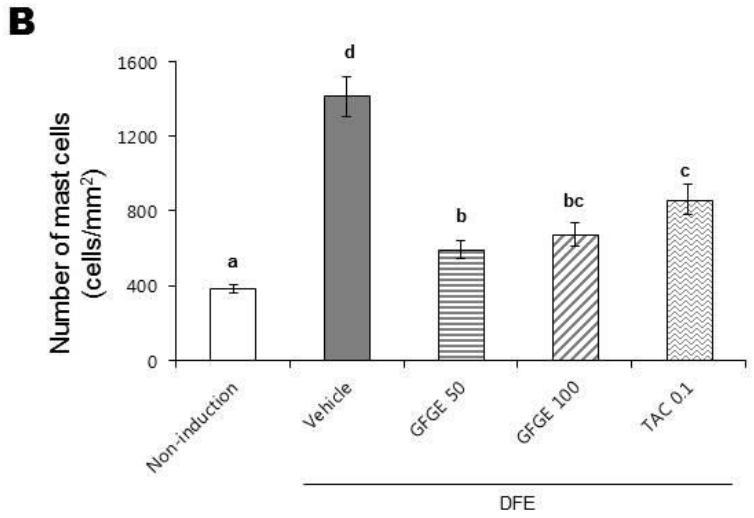

B
Number of mast cells (cells/mm²)
1600
1200
800
400
0
a
d
b
bc
c
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

Figure 5. Effect of GFGE on accumulation of mast cells in skin lesion.

A, Representative histological features of mast cells (toluidine blue (TB) staining) in skin lesion. Arrows indicate the TB-stained mast cells. The number of cells was counted under a microscope at 400× magnification. Scale bar: 50 μm. *B*, The number of mast cells in the 1 mm^2 of skin lesion. The data represents the mean ± SEM (n=7~8). Means with letters (a-d) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. GFGE 50, GFGE 50 mg/kg; GFGE 100, GFGE 100 mg/kg; TAC 0.1, tacrolimus 100 μg

3.3.5. Oral administration of GFGE reduced DFE-induced swelling of brachial lymph nodes in NC/Nga mice

Improvement of DFE stimulation by GFGE was also confirmed by the assessment for enlargement of brachial lymph node. The brachial lymph nodes in DFE with vehicle administered mice were markedly enlarged (Fig. 6A). The weights of drainage lymph nodes of DFE with vehicle administered mice were increased (14.92±1.42 mg), compared to non-induction group (6.90±0.55 mg). On the other hand, the weights of lymph nodes in GFGE 50, GFGE 100, and TAC 0.1 are 12.23±0.88 mg, 8.99±1.42 mg, and 14.94±0.81 mg. The major axis lengths of DFE with vehicle administered mice showed increased (4.56±0.17 mm), compared to non-induction group (3.23±0.08 mm). On the other hand, the lengths of lymph nodes in GFGE 50, GFGE 100, and TAC 0.1 are 4.48±0.15 mm, 3.73±0.02 mm, and 4.62±0.08 mm. Only GFGE 100 decreased the lengths of lymph nodes. Overall, I found that DFE-induced the swelling of brachial lymph nodes was suppressed by GFGE 100 administration, only.

Figure 6

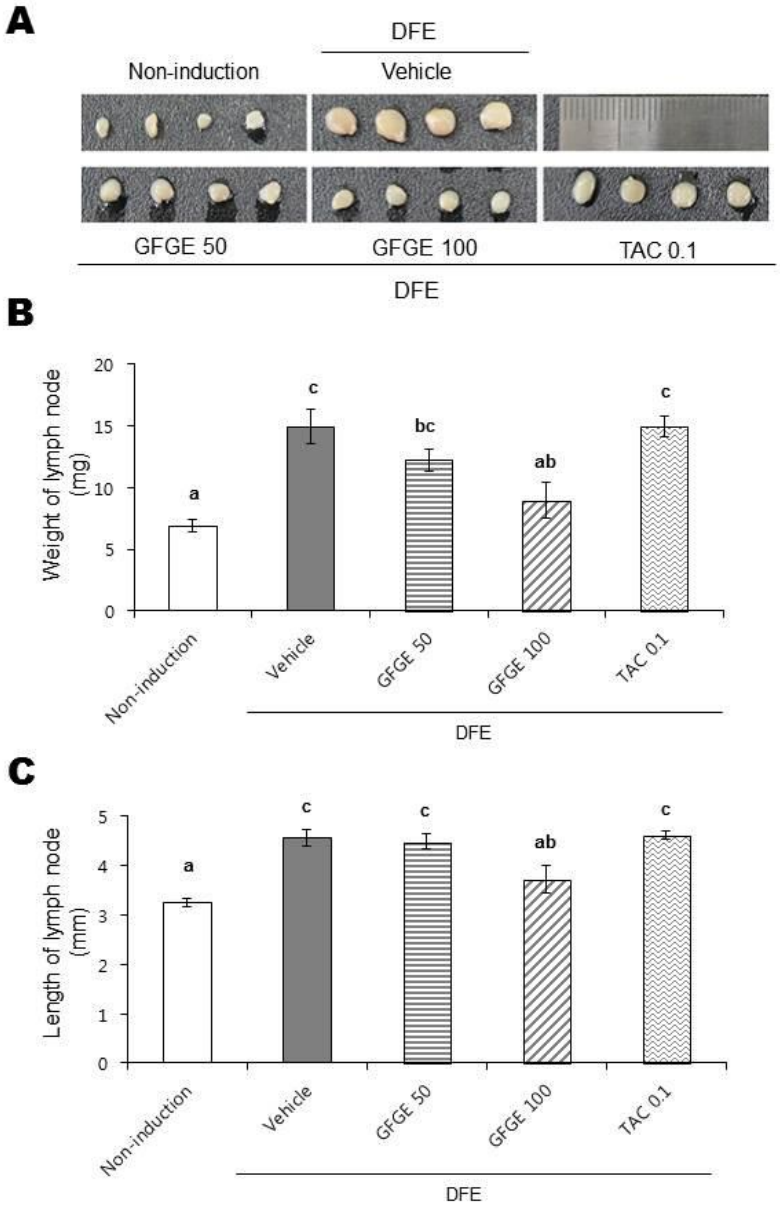

A
DFE
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE
B
Weight of lymph node (mg)
20
15
10
5
0
a
c
bc
ab
c
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE
C
Length of lymph node (mm)
5
4
3
2
1
0
a
c
c
ab
c
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

Figure 6. Effect of GFGE on stimulation of brachial lymph nodes.

A, Representative features of brachial lymph nodes. *B*, The weights of lymph nodes. *C*, The major axis lengths of lymph nodes. The data represents the mean ± SEM (n=7~8). Means with letters (a-c) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. GFGE 50, GFGE 50 mg/kg; GFGE 100, GFGE 100 mg/kg; TAC 0.1, tacrolimus 100 μg

3.3.6. Oral administration of GFGE regulated the levels of Th1/Th2 cytokine in cultured splenocytes and the level of MDC in serum

To examine the effect of GFGE in the production of Th1/Th2 cytokines, splenocytes derived from NC/Nga mice were stimulated with anti-CD3 and anti-CD28 mAbs. The level of Th2 cytokine, IL-5 was slightly increased in DFE with vehicle administered mice (42.81±15.27 pg per ml), compared to non-induction group (1.95±1.24 pg per ml). However, GFGE 50 significantly inhibited the production of IL-5 (2.53±1.81 pg per ml). This level showed the decreased tendency in other groups. The level of IL-13 was markedly increased by DFE administration (421.26±102.06 pg per ml), compared to non-induction group (100.15±7.56 pg per ml). However, GFGE 50, GFGE 100, and TAC 0.1 significantly reduced the level of IL-13 (113.89±2.00 pg per ml, 124.19±6.41 pg per ml, and 141.36±14.09 pg per ml). The level of Th1 cytokine, IFN-γ, also markedly increased by DFE administration (1919.48±430.31 pg per ml), compared to non-induction group (717.01±198.26 pg per ml). However, GFGE 50, GFGE 100, and TAC 0.1administration inhibited the level of IFN-γ (948.53±74.35 pg per ml, 769.74±114.27 pg per ml, and 705.70±117.90 pg per ml) (Fig. 7A, B and C).

Furthermore, I assessed the effect of GFGE on production of MDC level in serum. The level of MDC was markedly increased by DFE administration (109.79±5.77 pg per ml), compared to non-induction group

(38.09±8.87 pg per ml). However, GFGE 50, GFGE 100, and TAC 0.1 significantly reduced the level of MDC (68.86±2.40 pg per ml, 63.32±3.04 pg per ml, and 52.69±1.66 pg per ml). (Fig 7D) Overall, I found that the increased levels of cytokines and MDC by DFE-induced were reduced by GFGE administration.

Figure 7

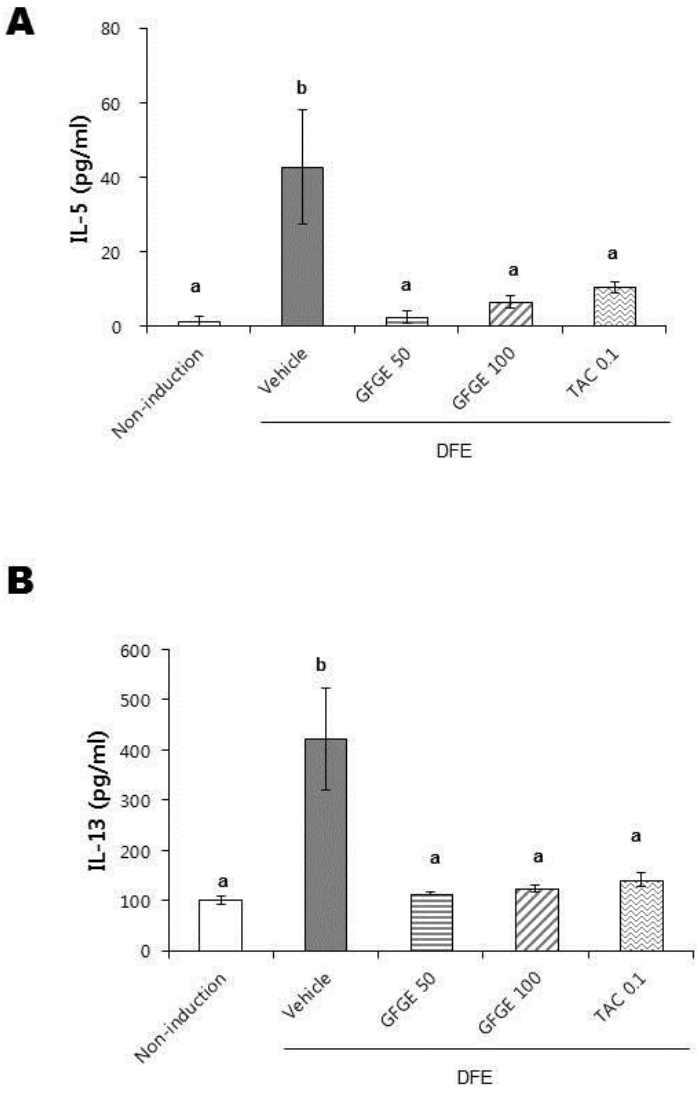
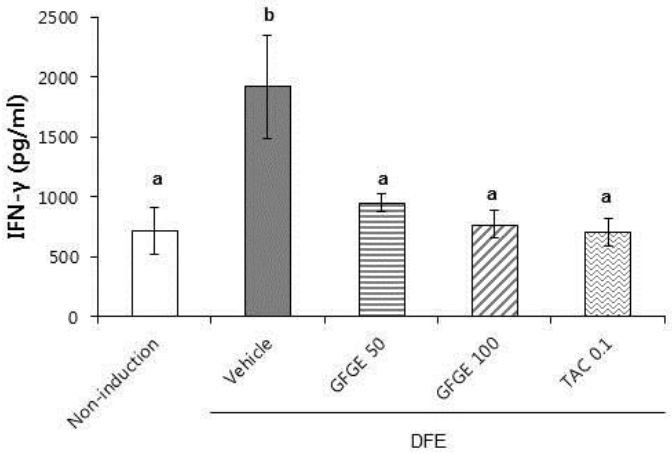

A
IL-5 (pg/ml)
80
60
40
20
0
a
b
a
a
a
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE
B
IL-13 (pg/ml)
600
500
400
300
200
100
0
a
b
a
a
a
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

Figure 7

C


IFN-γ (pg/ml)
2500
2000
1500
1000
500
0
a
b
a
a
a
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

D

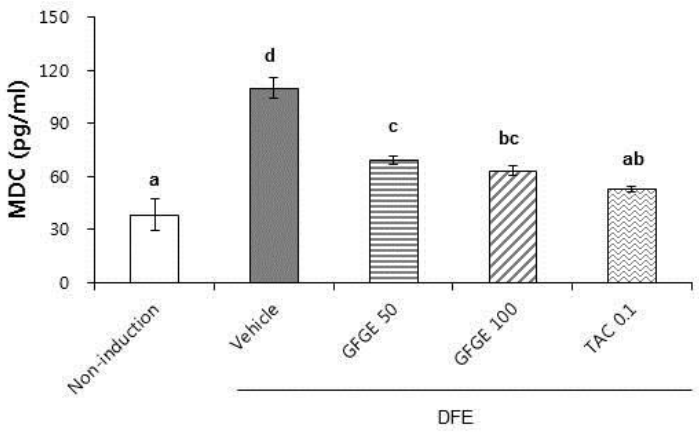

MDC (pg/ml)
150
120
90
60
30
0
a
d
c
bc
ab
Non-induction
Vehicle
GFGE 50
GFGE 100
TAC 0.1
DFE

Figure 7. Effects of GFGE on the production of Th1/2 cytokines in the cultured splenocytes and MDC in serum

Inhibitory activities of GFGE on the production of *A*, IL-5, *B*, IL-13, and *C*, IFN-γ were analyzed in splenocytes cultured supernatants which stimulated by anti-CD3 and anti-CD28 in 10% FBS RPMI1640 for 48 h. *D*, MDC was analyzed in serum. Cytokines and MDC levels were measured by ELISA. The data represents the mean ± SEM (n=7~8). Means with letters (a-d) within a graph are significantly different from each other at $P < 0.05$ as determined by Duncan's multiple range test. GFGE 50, GFGE 50 mg/kg; GFGE 100, GFGE 100 mg/kg; TAC 0.1, tacrolimus 100 μg

3.4. Discussion

NC/Nga mice were originated Japanese fancy mice (Nishiki-Nezumi) and established in 1955 [11]. Under the conventional environment, NC/Nga mice represent the skin inflammation and immunological skewing, highly resembles those of human AD [12]. However, the symptoms of AD are various and developed during long period, and if kept under the specific pathogen free environment, these symptoms are rarely developed [12]. Therefore, to reduce the variation, and induce effectively in a short-period, specific allergens are commonly used to induce of AD. House dust mites live in carpet, and bedding, and their body allergen is exposed to human continuously, and develop allergic disease including AD [6, 13, 14] *Dermatophagoides spp*. is the most common mite on humans, most AD patients have the high titers of specific IgE to *Dermatophagoides* mite [15, 16]. Therefore, to use *Dermatophagoides* farinae extract and NC/Nga mice for AD model are suitable and resonable to investigate the pathogenesis of AD or develop the therapeutic candidates of AD [17].

The AD skin lesions display the typical clinical features, erythema, hemorrhage, edema, excoriation, scaling and severe pruritus. The consistent scratching behavior disturbs sleep, and impairs the quality of life of both patients and their family [18, 19]. I found that GFGE reduced the clinical severities including scratching behavior. The thickening of the skin layer in

the lesions at DFE induced AD model was similar to AD patient. And it is well-known that eosinophils and mast cells play an important role in a chronic skin inflammation of patient with AD [20]. Histological examination showed that GFGE significantly reduced the hypertrophical changes, total number of infiltrated eosinophils and mast cells, similar with tacrolimus.

Topical administration of DFE causes the AD associated immune response, and then cytokine producing T cells are accumulated at the local lymph nodes [21]. The skin-draining lymph nodes proximal to the skin lesions exhibited massive enlargement elicited by accumulation of T cells. However, the enlargement was restricted to lymph nodes proximal to the skin lesions, whereas distal lymph nodes including inguinal and popliteal lymph nodes remained normal [21]. Therefore, assessment the enlargement of brachial lymph node might demonstrates the severity grade of immune response in AD model. I found that only GFGE 100 administration significantly reduced the weights and major axis lengths of brachial lymph node.

The upregulation of total serum IgE is the most common, and important feature of AD [5]. In this study, I assessed the level of total serum IgE four times, before inducing AD (day -21), before administration of GFGE (day 0), after 3 weeks of GFGE administration (day 21), and end of experiment (day 43). DFE explosively alleviated the level of total IgE since

day 0, the level of DFE with vehicle administration group exhibited 278.29±34.27 μg/ml at day 43. However, administration of GFGE and tacrolimus couldn't affect the level of IgE. The extremely high serum level of IgE have been reported during immunosuppressive therapy using tacrolimus . Because it had been reported to enhance some immune response, possibly through the action of tarcrolimus resistant immune system [22].

Cutaneous T cells that produce Th2 cytokines as IL-4, IL-5, and IL-13 predominate in the initial phase, whereas T cells that produce IFN- γ predominate in the chronic phase [23]. In general, although Th2 polarized immune response is explained the condition of AD, many studies demonstrates that Th1 cytokines (IFN-γ, IL-12) plays a critical role in determining the severity of chronic phase of AD lesions [24]. Th2 cytokines are reported to be markedly increased in AD. However, in this study, the level of Th2 cytokine were slightly increased especially IL-5. I thought that because increased Th1 cytokine, such as IFN-γ, suppressed the level of Th2 cytokines, and then neutralized in chronic phase of AD in this study. Although the levels of IL-5 were lowered, it displayed to significantly increase pattern, and GFGE 50 administration significantly inhibited the production of IL-5 in cultured splenocytes. Of course GFGE 100, and TAC 0.1 showed the inhibiting tendency for production of IL-5. On the other hand, IFN-γ was explosively increased by DFE administration, GFGE 50, GFGE

100, and TAC 0.1 suppressed it around normal level. Therefore, GFGE could suppress the severity of AD in chronic phase. The serum level of MDC have been reported that demonstrate the severity of AD symptom in patient with positive correlation [3]. In this study, the serum level of MDC was alleviated by DFE administration, GFGE 50, GFGE 100, and TAC 0.1 suppressed it significantly. I found that the improving effect of GFGE could also find in serum, it might be less invasive route compared to skin biopsy.

According to several epidemiological studies, approximately half of AD patients will develop asthma, and two thirds will develop allergic rhinitis [19]. AD is thought to trigger other allergic disease (Atopic march), and this phenomenon demonstrates the percutaneous sensitization through the impaired atopic barrier plays an important role for immunological imbalance [19]. Therefore preventing of development or well-management of AD in early stage is very important. The medicinal drug for treatment of AD, corticosteroids show very effective in almost AD patients, however, if it used for long time, it can cause severe side-effects; hyperglycemia, insulin resistance, osteoporosis, cataract, and skin atrophy. The calcineurin inhibitor, tacrolimus and pimeclorimus are known to be quite safe, but they have been reported to trigger skin cancer [25, 26].

In conclusion, these results demonstrate that oral administration of GFGE reduced dermatitis severities, scratching behavior, hypertrophic

change of epidermis and dermis, infiltration of eosinophils and mast cells, enlargement of brachial lymph node, and release of Th1/Th2 cytokines and MDC in prior developed AD model. Therefore I could suggest that oral administration of GFGE ameliorate the symptoms of AD in prior developed AD disease model.

3.5. References

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Chapter 4.

Conclusion

4.1. Conclusion

In the present study, I sought to investigate various effects that could provide further insights into the potential anti-allergic effects of GFGE. Results show that GFGE ameliorates the symptoms of atopic dermatitis. I found that GFGE can't affect the level of IgE, represent allergic response. However, in this study, tacrolimus also couldn't reduce the IgE level. Instead, ginseng is thought to be very effective for relieving pruritus. Although the oral administration of small amount of GPD (8 mg/kg) couldn't inhibit the dermatitis symptom, hypertrophy of skin, infiltration of inflammatory cells, and Th1/Th2 cytokines, however the scratching incidence was markedly reduced similar with GFGE and tacrolimus administration group (Data not shown). Although GFGE couldn't alleviate IgE-mediated allergenic response, pruritus, which crucial for quality of life, was significantly relieved.

GPD, which known to be most effective among ginsenoside, fortified ginseng extract was manufactured through specially designed biotransformation. And I investigated its anti-allergic effect and mechanism using AD model in mice. GFGE have the anti-allergic function, definitely. Although, I couldn't sought the effects of GFGE is not dose-dependently, low dose (50 mg/kg) of GFGE also improved the symptoms of AD.

Although these findings leave something to be desired to molecular

mechanisms, these results suggest that GPD fortified ginseng extract might be beneficial for immunobalancing. Further studies could be conducted to elucidate the molecular mechanisms, and investigate the effect on other allergenic diseases.

국문초록

아토피성 피부염은 건조증과 소양감을 주증으로 하여 환자에게 지속적인 고통을 주는 질환이다. 이는 생명을 위협하는 질환은 아니지만 지속적인 소양감으로 수면을 방해하고, 경제적인 부담을 줌에 따라 환자와 그 가족의 삶의 질을 크게 떨어뜨리는 질환으로 여겨지고 있다. 유전이나 서구화된 생활, 현대화된 식단, 환경 오염, 급속한 기후 변화 등으로 전 세계적으로 아토피성 피부염의 발병율이 증가하고 있으며 사회적, 경제적인 영향이 커짐에 따라 공중보건의 중요한 문제로 간주되고 있다. 아토피성 피부염은 Th1/Th2 사이토카인을 중심으로 면역불균형이 나타나는데, 초기에는 과도한 Th2 면역반응이 발생하다가 만성적으로 Th1 면역반응도 과도해지는 양상을 보이는 특징이 있다. 또한 건조증이나 긁는 행위로 인한 지속적인 피부장벽의 손상은 알레르겐의 침입을 촉진하여 아토피성 피부염을 심화시킬 수 있다.

아토피성 피부염은 대개 유·소아기에 발병하는 피부염으로써, 성장하면서 호전되지 않는 경우 외부 알레르겐의 피부 감작을 통해 다른 알레르기성 질환인 알레르기성 비염이나 천식 등의 발병을 촉진할 수 있다. 이러한 증상을 해소하기 위한 치료제가 개발, 적용되고 있지만 보습제를 제외하고는 장기간 사용 시 과한 면역부전이나 신독성, 피부암 등의 부작용을 발생시킬 수 있어 유아기부터 성인에 이르기까지 장기간 치료제를 사용한다는 것은 그 위험성이 크다고 하겠다. 그러므로 이러한 질환의 발

병을 예방하고, 지속적으로 관리하기 위해서는 면역조절, 염증완화 및 가려움증 등을 표적하는 천연물을 찾는 것이 중요하다.

인삼은 스트레스가 있는 환경에서 항상성을 유지할 수 있는 약초로 알려져 있으며 아시아 지역을 중심으로 4,000년 이상 쓰여왔다. 인삼 및 진세노사이드의 항알레르기 효과는 이미 많은 세포주 실험, 동물실험 등을 통해 제시되어 왔다. 진세노사이드가 장내에서 대사되어, 혈액으로 흡수, 이용될 수 있는 최종 형태인 글루코피라노실 프로토파낙사디올(GPD)은 가장 유효한 성분으로 알려져 있지만 일반적인 방법으로 추출한 인삼추출물에는 Rb 등 다른 진세노사이드만이 존재하며, GPD는 거의 함유되어 있지 않아, 섭취하는 사람에 따라 다른 정도로 대사, 흡수되게 된다. 본 연구에서는 특수한 공정을 통해 GPD가 8% 이상 존재하도록 생물전환을 한 GPD강화인삼추출물을 이용하여 이것의 항아토피성 피부염 효과를 마우스 모델에서 확인하였다. 이는 예방효과를 탐색하기 위한 연구로서 아토피성 피부염을 유도하기 이전부터 복용시킨 경우와, 치료적 완화효과를 탐색하기 위한 연구로서 아토피성 피부염이 발생된 이후부터 복용시킨 경우 모두에서 그 효과가 입증되었다. 아토피성피부염의 예방적, 치료적 완화효과는 가려움증을 완화하고, 병변에서 호산구와 비만세포의 침윤을 줄였으며, Th1/Th2 사이토카인을 비롯한 다양한 사이토카인과 케모카인의 분비를 조절하는 것을 확인함으로써 입증되었다.

종합적으로 아토피성피부염을 예방하거나 완화할 수 있도록 지속

적인 관리가 필요하며, 그에 있어 본 연구를 통해 면역조절능이 확인된 GPD강화인삼추출물이 효과적인 예방적, 보조적 치료제로 사용될 수 있을 것임을 시사한다.